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Impact of water relations on the production, ecological fitness and stability of the biocontrol fungus *Metarhizium anisopliae*

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ABSTRACT

The aim of this work was the production of high numbers of propagules of the entomopathogenic fungus *Metarhizium anisopliae* with high germination capacity under low water availability, good storage stability and enhanced pathogenicity. To this end modifications of cultural conditions were employed. Water-stress in solid substrate fermentation reduced the conidial production but resulted in enhancement of germination capacity. Conidial production and germination was also considerably influenced by the type of solid substrate (bulgar wheat, millet grain). Intracellular accumulation of endogenous reserves of selected polyols, which have been linked with enhancement of germination under conditions of restricted water availability, was altered by such substrate modifications. Imposed water-stress in liquid culture resulted in increased blastospore production in the 0.998 to 0.96 water activity (a_w) range when ionic solutes (NaCl, KCl) were used for medium modification but when PEG 200 was used, this range was narrower (0.998-0.98 a_w). Water-stress resulted in the production of modified blastospores with increased levels of the low molecular weight polyol erythritol. Higher amounts of endogenous polyols were retained intracellularly when modified blastospores were harvested in isotonic solutions compared to those subjected to hypo-osmotic shock by washing with water. Intracellular polyol patterns of blastospores were also affected by nitrogen source, pH and a_w-modifying solute as well as by interaction between these factors. Optimum conditions for increased erythritol accumulation occurred when ionic solutes (NaCl, KCl) were used for a_w modification and in the pH range between 6.8-8. Total endogenous protein was also enhanced under the same conditions. Germination of blastospores produced under these conditions was between 62-89% under conditions of water-stress (0.96 a_w). In contrast, blastospores with lower amounts of erythritol and total protein content had decreased germination (8-67%). Osmoprotection of such modified blastospores resulted in increased storage stability as wet pastes and as freeze-dried preparations. Fluidised bed dried blastospores had either decreased viability (<25%) or good viability (>40%) but high conent of moisture (>30%). Destruxin A production was not affected by osmoprotection whereas protease activity was enhanced by isotonic washing with PEG 200 solution but not by ionic (NaCl) or carbohydrate (glucose) solutions. Modified blastospores were not more infective to aphids than unmodified ones under 100% R.H. regimes.

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Chapter 1 LITERATURE REVIEW

1.1 INTRODUCTION

It is now generally recognized that chemical pesticides can have an impact on operators, consumers, a wide range of organisms, contaminate groundwater and enter food chains (Moore & Prior, 1993). These concerns, in conjunction with the fact that resistance to chemical pesticides is increasing, have provided strong incentives for alternative more environmentally friendly methods for the control of insect pests. Insect growth regulators, insecticidal soaps, horticultural oils, predators and parasites, and microbial control agents such as viruses, nematodes and fungi are all potential alternatives to chemical pesticide strategies. An inexperienced viewer would easily think that with such a plethora of choices, the problem of chemical pesticide replacement could easily be resolved. Unfortunately, in reality it is not so simple. For a new pest control agent to be successfully developed and implemented, many issues need to be addressed. Economic, ecological and social considerations, efficiency, consistency, stability in storage and ease of use are important considerations.

Fungi possess certain attributes that make them attractive candidates as biological control agents (BCA); they are relatively easy and cheap to produce in comparison to other BCAs and if used carefully, they do not pose any risks to animal and human health. The area of fungal biological control is a continuously developing one which includes a number of important disciplines, such as pathology, ecology, genetics, physiology, mass production, formulation and application strategies.

1.2 BENEFITS AND PROBLEMS IN DEVELOPING FUNGI AS BCAs

Production of fungal BCAs can be easily achieved using inexpensive culture systems based mainly on solid state fermentation using cheap agricultural products (Goettel,

1984) or in liquid fermentation (Bidochka *et al.*, 1987; Kleespies & Zimmermann, 1992; Jackson *et al.*, 2003). This ease of mass production makes fungi successful candidates for the development of an economically viable biopesticide. Fungal BCAs are naturally occurring microorganisms, and when used carefully after an appropriate screening programme to determine any potential risk for non-target organisms, they are very safe and do not have any harmful effects on the environment.

The wide-spread use of fungal BCAs as a method of controlling insect pests in agriculture will result in the production of agrochemical-free products or at least in a reduction in chemical input and may thus contribute to an improved welfare of humans and animals. Such a wide use may also contribute to increased income for the grower because of the premium on pesticide-free and organic produce.

Unfortunately, there are some important limitations in the use of fungal BCAs that need to be overcome. First of all they need to compete with the broad-spectrum insecticides, the overall development cost of which is often lower (the relative returns are bigger). They are also more host specific than chemical insecticides/pesticides and thus they are aimed at small niche markets making it even more difficult to be financially viable. The need for toxicity and safety studies similar to that of chemicals adds more financial costs for their commercialisation. Lastly, the introduction of a pest control method using fungal BCAs will require re-education of growers and provision of good information to the public for their acceptance. The restrictions on the use of fungal BCAs is discussed in more detail in Section 1.9.

1.3 FUNGAL PATHOGENESIS OF INSECTS

Before starting the description of fungal pathogenesis of insects, it is useful to distinguish the difference between pathogenicity and virulence. Pathogenicity is the qualitative ability of a pathogen to cause disease, whereas virulence is the quantitative amount of disease that a pathogen can incite in a group of insects. Therefore, it is possible to have an avirulent isolate of a pathogenic species with respect to an individual host.

Very early studies speculated that the mechanism of fungal infection of insect hosts was through ingestion of the pathogen (Samson *et al.*, 1988). It has been shown that most entomopathogenic fungi invade insects by breaching the host cuticle (Hajek & St. Leger, 1994). This mode of infection distinguishes fungi from other insect pathogens, like bacteria, viruses and nematodes, which penetrate their host via the mid-gut.

The development of an insect mycosis can be divided into three phases:

(i) adhesion and germination of the spore on the insect cuticle

(ii) penetration of the insect integument by a germ tube

(iii) development of the fungus inside the insect body which usually results in death of the host (Samson *et al.*, 1988).

1.3.1 Attachment of the spore to the cuticle

The insect cuticle is the host tissue that entomopathogenic fungi initially contact. Firstly, for a successful infection process to occur, the fungal spore must adhere to the insect cuticle and recognise a susceptible host. The physical and chemical structure of both the outer layer of the insect integument and the outer layer of the fungal spore wall play a significant role in the adhesion and recognition process (Samson *et al.*, 1988; Boucias & Pendland, 1991; Hajek & St. Leger, 1994). For example, the hydrophobic conidia of *Metarhizium anisopliae* and *Beauveria bassiana* possess an outer rodlet layer which in addition to providing protection against dehydration and aiding in the aerial dispersal of conidia, may have a major role in the attachment of conidia to the host cuticle which is also hydrophobic (Boucias & Pendland, 1991). Additionally, while conidia of these species were capable of attaching to all body regions, more spores were attached to cuticle surfaces containing short cuticular spines indicating the effect of cuticle topography on spore attachment (Boucias *et al.*, 1988). Insect pathogenic fungi producing hydrophilic conidia typically lack a well-organised outer rodlet layer but they possess, in most cases, a mucus coat that facilitates their adhesion to the host cuticle (Boucias & Pendland, 1991).

Fungi also secrete small proteins, so-called hydrophobins, which are involved in formation of hydrophobic aerial structures like aerial hyphae, spores and fruiting bodies

and mediate attachment of hyphae to the hydrophobic surface like the insect cuticle (Wösten & Vocht, 2000). Carbohydrate-binding proteins have also been detected in the conidial wall of various entomopathogenic fungi such as *Nomuraea rileyi*, *B. bassiana* and *M. anisopliae* but has not been proven to have a direct effect on spore attachment to the cuticle (Boucias & Pendland, 1991).

As far as terrestrial fungal entomopathogens producing hydrophobic conidia are concerned, adhesion of the fungal propagule to the arthropod cuticle involves passive, non-specific mechanisms as a result of strong hydrophobic forces and to a lesser extent electrostatic interactions (Boucias & Pendland, 1991). However, certain components present on fungal spore walls have been proposed to confer recognition for different hosts. For example, antigenic determinants found on the spore surface of *M. anisopliae* have been correlated with virulence to the subterranean pasture scarab *Adoryphorus couloni* and it has been proposed that the specific structure of these antigens could confer differing recognition for different hosts (Rath *et al.*, 1996). Physico-chemical cues on flea beetles have also been suggested to encourage or reduce conidial adhesion. Butt *et al.* (1995) found that more conidia of *M. anisopliae* adhered to the cuticle under the elytra of the flea beetle compared with sites on the dorsal elytra and ventral thorax. Poor adhesion was not observed in dead beetles. The surface topography, the chemical properties of the host cuticle as well as components derived from plant, substrate, host faecal material, or from microbial flora present on the cuticle surface may all influence conidial adhesion and subsequent stimulation and growth of penetrant germ tubes (Boucias & Pendland, 1991). Therefore the developmental stage and the plant host of an insect may affect its susceptibility to pathogenic fungi.

1.3.2 Germination of the spore

After attachment of the spore to the insect, and providing that the factors that influence pathogenicity, such as water availability, temperature, and nutrition (which are referred to in a following section) are favourable, the spore will germinate and penetrate the insect cuticle either directly or via a penetration peg produced from under an appressorium. Many fungi have been found to secrete mucilage during germ tube and/or appressorial formation which may consolidate the spore attachment to the host cuticle

(Bidochka *et al.*, 1997). Furthermore, due to its hydroscopic nature it may create a favourable environment for the extracellular enzymes released by these structures (Boucias & Pendland, 1991). Physico-chemical cues on the host cuticle already referred to affecting the fungal spore attachment (see Section 1.3.1), may also influence appressorial formation. *M. anisopliae*, for example, produces fewer appressoria on the soft aphid cuticle compared with beetle cuticle suggesting that penetration is more direct on soft bodied insects (Butt *et al.*, 1995). An array of factors can influence germination of a fungal spore on an insect cuticle including the availability of nutrients (endogenous to the cuticle, exogenously deposited, or host exudates), presence or absence of compounds inhibitory to germination on the outermost surface of the cuticle, and environmental factors such as relative humidity, temperature and UV radiation (Bidochka *et al.*, 1997). However, the crucial bottleneck for the germination of most fungal biocontrol agents on the insect cuticle surface is water availability. Most fungal biocontrol agents require at least 95% relative humidity in order to germinate (Gillespie & Crawford, 1986), a condition which does not often prevail in the field for long periods of time.

Improvement in fungal spore germination (with *M. anisopliae* as the model microorganism) under low water availability levels together with methods of production of high spore yields in the shortest possible fermentation time and using cheap raw materials, represents a major part of this work. The time taken between spore germination and host penetration plays an important role in the virulence of a entomopathogenic fungus. The longer a spore remains ungerminated, the more likely it is that it will be exposed to adverse environmental conditions. Quantitative and temporal differences in adhesion and germination have often been related to pathogenicity (Jackson *et al.*, 1985; Samuels *et al.*, 1989; Hassan *et al.*, 1989).

1.3.3 Penetration of the host integument

Successful germination on the host cuticle does not always lead to infection. Several factors such as environmental, physiological and/or morphological factors of the host can prevent pathogenesis after germination. Successful penetration of the tough integument should involve mechanical and enzymatic activities in the developing germ

tube as evidenced by the presence of shallow depressions and zones of histolysis at the site of penetration (Samson *et al.*, 1988; St. Leger *et al.*, 1989). The composition of insect cuticle is primarily protein, followed by chitin and lipids (Locke, 1984). Therefore enzymatic degradation of these compounds could help the invasion of a fungal pathogen through the cuticle. Various enzyme activities have been detected in pregerminating conidia as well as in the mucilage produced by germ tubes and appressorial cells (Boucias & Pendland, 1991).

Many pathogen enzymes have been correlated with virulence of fungal pathogens. For example, a cloned protease (Pr1) from *M. anisopliae* solubilizes cuticle proteins, thus assisting penetration and nutrient provision for further growth (St. Leger *et al.*, 1992). However, whether or not this enzyme is a determinant of insect pathogenicity is questionable since stable mutant strains of *M. anisopliae* lacking *pr1* genes are still able to infect insect hosts albeit with reduced virulence (Wang, *et al.*, 2002). Similarly, virulence of *Verticillium lecanii* has been linked with high extracellular chitinase activity (Jackson *et al.*, 1985). However, high levels of *in vitro* produced enzymes are not always correlated with virulence of a fungal strain and care must be taken in interpretation of these results. There are several reasons for a non-direct relationship between high enzyme production and virulence of a fungus: (i) The speed of enzyme production is more important than the amount of the enzyme produced and only limited amounts of enzyme production are required for successful pathogenicity, (ii) *in vitro* produced enzymes may differ qualitatively and quantitatively from *in vivo* produced enzymes and (iii) non-pathogenic strains may produce the same type and amount of enzyme as the pathogenic ones but they may be unable to utilise the released monomers for their nutrition and subsequent growth (Samson *et al.*, 1988).

1.3.4 Development of the fungus inside the host

Once fungi penetrate the host integument, penetrant hyphal buds develop as blastospores within the haemocoel (Hajek & St. Leger, 1994). The invading pathogen employs an array of biochemical and morphological changes in order to avoid/overcome any host defence reactions and exploit the nutritional environment in the heamocoel at the expense of the host. Enzymes are secreted or constitutively

induced which break down compounds, mainly trehalose (the most abundant carbohydrate in the haemolymph) and organic phosphates, providing the pathogen with nutrients for its growth (Xia *et al.*, 2001; 2002). Apart from nutrient provision, enzyme production during mycosis can lead to an accumulation of glucose in the haemolymph, which may function as a feedback mechanism controlling the initiation of feeding and subsequent insect starvation (Xia *et al.*, 2002).

It has also been found that many entomopathogenic fungi produce toxins during their invasion in the host, but only a few have been studied for their correlation with pathogenicity. The most studied toxins of entomogenous fungi in relation to insect pathogenicity are the cyclic depsipeptide destruxins produced mainly by isolates of *M. anisopliae*. The relative importance of these mechanisms as determinants of virulence is dependent on the specific fungal isolate or host. Two virulence strategies have been proposed; the "toxin strategy" and the "growth strategy" (Valadares-Inglis & Peberdy, 1998). Strains of *M. anisopliae* that produced destruxins *in vitro* and *in vivo* grew sparsely within the haemolymph of infected caterpillar larvae *Manduca sexta* and caused paralysis prior to death whereas no or low producing strains grew profusely in the haemolymph (Samuels *et al.*, 1988). Since fungal pathogenesis is a complex phenomenon, it is impossible at present to distinguish which strategy is the most efficient and recent studies showed that fungal strains with high production of destruxins can have the same LT_{50} (4 days) as low destruxin producing strains (Amiri-Besheli *et al.*, 2000). Destruxins affect several cellular organelles, such as mitochondria, endoplasmic reticula and nuclear membranes, causing dysfunction in the insect host (Samuels *et al.*, 1988). Other toxins include the cyclic depsipeptides beauvericin, bassianolide and the red-coloured dibenzoquinone oosporein, which are produced by *Beauveria* spp. A good review of entomopathogenic toxins can be found in Vey *et al.* (2001).

1.4 HOST DEFENCES AGAINST FUNGAL INFECTION

As soon as fungal germination on an insect host begins, the resulting germlings are continually moving through different environments and may encounter various host

defences. The resistance of insect hosts to the fungal invaders may result from the independent or combined effect of passive (constitutive) barriers and active (induced) mechanisms. This resistance can be encountered at any stage of disease development. Fungal pathogens respond to these defences by invoking adaptive biochemical processes and produce different infection structures possibly as a result of the evolution of a mechanism by which entomopathogenic fungi overcome host barriers (Hajek & St. Leger, 1994).

1.4.1 Host defences prior to and during penetration of the host cuticle

During the first steps of disease development, the host resistance mechanisms involve mainly passive, pre-existing barriers. First of all the outer epicuticle, although fragile, may provide a level of resistance to enzymatic degradation (Locke, 1984). The inner epicuticle, on the other hand, which is composed of polymerised lipoprotein stabilised by quinones, is susceptible to enzymatic degradation but its toughness provides some mechanical resistance to pathogen invasion. Additionally the lipid fraction of the epicuticle, whose main constituent parts are lipids, lipoproteins, polyphenols and proteins, has been reported in some cases to inhibit germination or to result in the production of non-penetrant germ tubes while in others to stimulate germination (Boucias & Pendland, 1991). The procuticle, which follows the epicuticle and is the main part of the cuticle, is composed of proteins complexed to chitin fibrils. This structure confers mechanical resistance to pathogen invasion (Hajek & St. Leger, 1994).

Physical damage of the host cuticle or β-1,3 glucans present on the fungal cell wall can induce cuticular melanization (Hajek & St. Leger, 1994). Insect phenoloxidases present in the cuticle and in the haemolymph have been implicated in melanic patches on wounded acridid cuticles or fungal infection sites (Gillespie *et al.*, 1991). Phenoloxidases have been found to be induced by β-1,3 glucans by fungal cell walls (Ashida *et al.*, 1983). The phenolic oxidation products and melanin may chemically insulate the hyphae by restricting the diffusion of enzymes and toxins from the fungus while at the same time limiting the availability of nutrient, water and oxygen (St. Leger,

1991). However, melanization often occurs too late or too little to stop fast growing or the more virulent pathogens.

1.4.2 Host defences after penetration of the host cuticle

After penetration of the host cuticle, fungi proliferate within the body of the insect and the outcome of the disease they cause is dependent on the pathogen's genetic potential to grow rapidly, to penetrate host-induced barriers and to resist toxic chemicals. Although arthropods have a primitive immunological system, they are able to recognise the non-self and react to the entrance of a fungal pathogen inside their body. Defence mechanisms can be cellular and/or humoral (Samson *et al.*, 1988).

The main reaction in the cellular antifungal defence mechanism is encapsulation of the fungal element following initial recognition of the fungus by haemocytes (Hajek & St. Leger, 1994). Encapsulation involves recruitment of granulocytes, which are attracted to the fungus and may engulf it, then plasmatocytes recruitment which form a pseudotissue in concentric layers forming a granuloma in which the fungus may be lysed (Hajek & St. Leger, 1994). The prophenoloxidase activating system, which is located in the granulocytes, becomes activated during encapsulation and converts to phenoloxidase resulting in melanization of the fungal propagule (Samson *et al.*, 1988). The encapsulation trigger is suggested to involve a response to β-1,3 glucans present in the fungal cell wall (Huxham *et al.*, 1989). Encapsulation provides protection against only weakly virulent pathogens; hypervirulent strains are able to overcome the encapsulation and continue to grow or hosts are unable to form typical granulomas against these strains (Samson *et al.*, 1988; Hajek & St. Leger, 1994).

Fungi have developed several mechanisms to overcome the insect defence reactions. Destruxins, which can play a role in fungal virulence, may also interfere with the immune host response. It has been reported that destruxins impaired haemocyte aggregation and phenoloxidase activation by β-1,3 glucans in the desert locust *Schistocerca gregaria* (Huxham *et al.*, 1989). This group of toxins cause dysfunction in the insect host particularly at the mitochondrial or endoplasmic reticulum levels and as a consequence they can weaken the insect cellular reaction. Another strategy employed by

entomopathogenic fungi to evade recognition by host haemocytes is to alter the fungal cell-wall composition so that it exposes less β-1,3 glucans. For example, the hyphal bodies of *B. bassiana* in larval *Spodoptera exigua* (Lepidoptera) haemolymph, have different cell-wall properties from those grown in Sabourauds dextrose broth (Pendland *et al.*, 1993). Several species of Entomophthorales can spontaneously form protoplasts in the haemocoel of the insect and are able to grow in this protoplast stage (Samson *et al.*, 1988). The protoplasts do not expose β-1,3 glucans on their membranes and thus avoid recognition by the haemocytes. Another way by which fungi can escape the defence reaction of the insect host is by quick multiplication of infective propagules. A resistant insect will stop being resistant if the dose of invading propagules is very high. Most entomopathogenic fungi, especially hyphomycetes, form yeast-like propagules which are quickly disseminated throughout the blood stream making it difficult for the haemocytes to engulf all of the fungal elements (Samson *et al.*, 1988).

As far as humoral host defence reactions are concerned, most published accounts involve a complex of inducible, fungitoxic protease inhibitors (Hajek & St. Leger, 1994). Vilcinskas & Wedde (1997) studied the development of *B. bassiana* in the haemolymph of the moth larvae *Galleria mellonella* and strongly suggested that the capacity of insects to release inhibitors against fungal proteases influences their susceptibility against entomopathogenic fungi. Other humoral defences, such as antimycotic activity of lysozyme, have also been proposed (Vilcinskas & Matha, 1997).

Specific behaviours of some insects can also prevent lethal fungal infection. For example, the grasshopper *Camnula pellucida*, basks in the sun raising its internal body temperature above the optimum for development of *Entomophaga grylii* and in this way they recover from infection (Carruthers *et al.*, 1992).

1.5 FACTORS INFLUENCING EFFICACY OF FUNGAL BIOLOGICAL CONTROL AGENTS

1.5.1 The pathogen

The most important pathogenic factor affecting its efficacy in disease development is its physiology (e.g. production of enzymes and toxins), because this greatly affects the ability of a pathogen to incite disease. Fungi have one of the widest host ranges among the pathogens of arthropods. However, host spectra vary widely depending on fungal species and individual isolates within certain taxa can have a substantially restricted host range (e.g. *B. bassiana*, *M. anisopliae*) (Inglis *et al.*, 2001).

Apart from pathogen physiology, other factors influencing efficacy is the propagule density which must be sufficiently high to ensure insect contact by an adequate number of propagules (inoculum threshold). The ability of an entomopathogenic fungus to persist in an environment is another important characteristic for its success as a BCA. The longer the persistence of fungal propagules the higher the possibility of an insect coming in contact with sufficient propagules to cause disease. It must be borne in mind however, that inoculum threshold is dynamic and it is influenced by environmental conditions as well as by factors relating to insect hosts. For example, as already mentioned, grasshoppers bask in the sun raising their body temperature above optimum for fungal development and in this way disease development is avoided under such conditions even if inoculum is present at high concentrations (Carruthers *et al.*, 1992; Inglis *et al.*, 2001; Blanford & Thomas, 2001).

1.5.2 The insect host

One of the most important host factors influencing insect susceptibility to entomopathogens is stress (Inglis *et al.*, 2001). While a variety of factors are believed to stress insects and make them susceptible to fungal infection (e.g. crowding, nutrition, exposure to chemical stressors, environment), the physiological mechanisms of stress are inadequately understood. Diet can increase or decrease the susceptibility of insects to pathogenic fungi. For example, a poor diet can lead to reduced growth of insects with concomitant decrease in disease resistance. On the other hand insects may derive

fungitoxic compounds from their diet rendering them more resistant to fungal infections (Ekesi *et al.*, 2000). Other factors such as the developmental stage, insect density and several behavioural traits can have an effect on insect susceptibility to disease development by fungi.

1.5.3 The environment

Environmental factors that can influence biocontrol efficacy of entomopathogenic fungi include solar radiation, temperature, water availability and rainfall. Although each individual environmental factor can have a profound effect on fungal efficacy, the interaction between environmental factors should also be considered.

Solar radiation, and in particular the UV-B portion of the solar spectrum can adversely affect conidial culturability and germinability and therefore persistence in the field (Alves *et al.*, 1998; Braga *et al.*, 2001). Fungal propagules can be highly susceptible to damage by solar radiation but the degree of susceptibility depends on the strain and the microhabitat in which a fungus exists.

Temperature is of course one of the most important factors influencing entomopathogen efficacy. Different fungal strains have different temperature optima for their germination, growth and sporulation, and thus temperature can affect the time course of the disease development by fungi (Inglis *et al.*, 2001).

Water availability is the most crucial factor affecting the efficacy of a fungal BCA and given that sufficiency of water is an absolute requirement, other factors can be conceived as having a regulatory role. Apart from the effect that water availability can have on the persistence of fungal propagules, its main effect on the efficiency of entomopathogenic fungi, is centred on the germination and disease development by these microorganisms. Although the dependence of fungal germination and subsequent growth can range widely from complete dependence on free water to independence of an exogenous supply, most entomopathogenic fungi require high water availability (usually at least 95% RH) to germinate and infect their hosts (Hallsworth & Magan, 1999). However, this dependence of entomopathogenic fungi on high water availability

is mainly restricted on the insect surface since the prevailing water availability in the host haemolymph is usually relatively high and within the limits for their optimum growth. Additionally, ambient humidity is not always critical for the efficacy of entomopathogenic fungi, and germination as well as host infection can occur under lower ambient humidity if sufficient moisture exists within the microhabitat of the fungus-insect system. It has been found, however, that oil formulations of fungal spores enhance efficacy of spores at low relative humidity, so entomopathogenic fungi can be used under climatic conditions, which are less suitable for these antagonists (Bateman *et al.*, 1993; Kooyman & Godonou, 1997). Insufficient germination of fungal propagules under stress conditions of low water availability is considered one of the most important bottlenecks in the success of entomopathogenic fungi and represents a major part of the present study. The mechanisms employed by fungi under conditions of low water availability to retain their viability as well as the methods used to manipulate this ability and increase their efficacy, are discussed in the following Section.

1.6 MECHANISMS EMPLOYED BY FUNGI UNDER CONDITIONS OF LOW WATER AVAILABILITY

1.6.1 The concept of water activity and water potential

Scott (1957) was first to suggest the term water activity (a_w) to describe the water availability for microbial growth. Total water content of a substrate is not always a good indicator of water availability for microbial growth. For example the water content of a solid substrate consists of strongly bound water and free weakly bound water. Free water is more readily available for microbial growth and how easily this can be removed depends on the water content and the type of the substrate. Water activity is defined by the ratio between the vapour pressure of water in a substrate (P) and the vapour pressure of pure water (P_0) at the same temperature and pressure. Thus:

$$a_w = \frac{P}{P_o}$$

The a_w of pure water is 1 and any substrate with no free water will subsequently have an a_w less than 1.

Water potential (Ψ_w) is an alternative measure to a_w and is defined as the amount of work that must be done per unit quantity of pure water in order to transport reversibly and isothermally an infinitesimal quantity of water from a pool of pure water at atmospheric pressure to a point in a system under consideration at the same point elevation (Griffin, 1981). Ψ_w is the sum of the pressure potential (Ψ_p, the potential of water per unit volume as affected by external pressure; in filamentous fungi it represents the turgor potential of the protoplasm created by plasma membranes and cell walls), solute (or osmotic) potential (Ψ_s, the potential of water per unit volume as affected by the presence of solutes), and matric potential (Ψ_m, the potential of water per unit volume as affected by the presence of a solid matrix). Thus,

$$\Psi_w = \Psi_p + \Psi_s + \Psi_m$$

Ψ_w is related directly to a_w by the following formula:

$$\Psi_w = \frac{RT}{V_w \ln a_w}$$

where R is the ideal gas content, T the absolute temperature and V_w is the volume of 1 mole of water. The advantage of Ψ_w is that it is possible to partition osmotic and matric components and their influence on growth and physiological functioning of microbes. Soil microbiology studies use water potential, while for solid substrates, where solute potential is the major force, a_w is commonly used. The relationship between a_w and Ψ_w is shown in Table 1.1.

Table 1.1. Equivalent water activity, equilibrium relative humidity and water potentials at 25°C (Magan, 1997).

Water activity	E.R.H. (%)	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.30
0.60	60	70.30

1.6.2 The concept of osmosis and its importance in the absorption of environmental water by fungal spores

Osmosis is defined as the net movement of water molecules from a region where their concentration is high to a region where their concentration is low, through a partially permeable membrane. This is the process involved in the movement of environmental water into the fungal spores, the partially permeable membrane in this case being the fungal spore membrane. Therefore, water will be available to a fungal spore when its internal water potential is lower than that of the external environment. Even if all the necessary requirements for fungal spore germination initiation are present, germination will not start if water is not available to the spore. The term "necessary requirements for fungal spore germination initiation" includes factors such as nutrients, temperature, light wavelength, presence or absence of inhibitory compounds and microbial antagonism. It is very useful to mention here that fungal spores fall into two categories depending on whether or not they require exogenous supply of nutrients in order to germinate. Thus, they may be described as "nutrient sufficient" if they require only

water and oxygen to germinate or "nutrient insufficient" if an exogenous supply of nutrients is necessary for spore germination initiation. The entomopathogenic fungus studied in this work fall into the latter category (Dillon & Charnley, 1990). Figure 1.1 presents diagrammatically how water relations between intracellular and external environment lead to water absorption and subsequent germination of a fungal spore, providing that all the requirements for fungal germination are fulfilled.

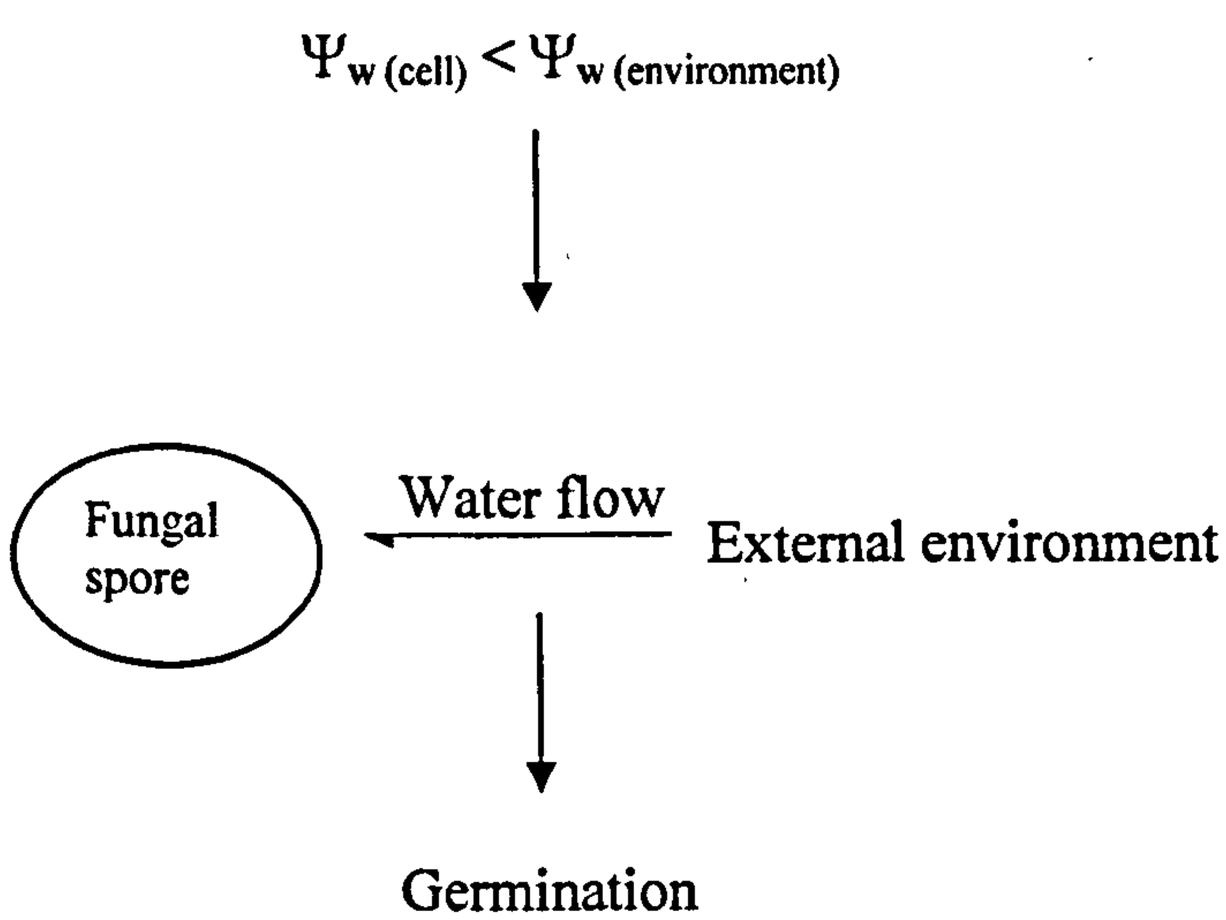


Figure 1.1. Diagrammatic representation of prerequisite water relations between intracellular and external environment of a fungal spore for germination initiation.

Considering the high impact of water availability on the efficiency of fungal propagules as BCAs (see Section 1.5.3), the mechanisms involved in adaptation to low water availability levels and their manipulation during production of a fungal BCA are of particular importance.

1.6.3 Adaptation to low water availability: the role played by polyols

At low water availability fungi have been shown to accumulate polyols (acyclic sugar alcohols) to high concentrations reducing intracellular water potential (Brown 1978; Adler *et al.*, 1982; Hallsworth & Magan, 1994a,b; Pascual *et al.*, 2000a; Frey & Magan 2001), while at the same time preventing enzyme inhibition due to dehydration (Carpenter & Crowe, 1988). In this way water is still available to fungi at low water availability maintaining their turgor and thus enabling them to carry on cell functioning efficiently. However, clear evidence for a major contribution of polyols to intracellular

water potential comes mainly from studies on yeasts. Abadias *et al.* (2001) presented work showing a high correlation between total polyol accumulation and intracellular water potential adjustment in the biocontrol yeast *Candida sake*. Some fungi have been reported to synthesise the amino acid proline instead of polyols when grown on media containing either ionic or non-ionic solutes to modify water potential (Luard, 1982c). Both polyols and proline are compatible with fungal metabolism at high concentrations (they do not destabilise or inhibit enzymes) and are referred to as compatible solutes as first defined by Brown & Simpson (1972).

Apart from maintenance of fungal turgor and protection of enzymes from denaturation, polyols can also serve other roles. They can play a metabolic role in fungi and be involved in energy dissipation through their interconversion with another compound, the so-called futile cycles (Jennings & Burke, 1990). In this way energy is spent through these cycles without corresponding changes in the concentrations of the compounds involved. This "energy spilling" regulates the overproduction of ATP at the expense of ADP under carbon excess conditions, which leads to the production of overflow metabolites and reducing equivalents. The excess of reducing equivalents produced under such conditions can also be regulated by polyols driving in this way oxidative and reductive reactions. For example, the formation of mannitol from glucose consumes reducing equivalents (Jennings & Burke, 1990). Since polyols drive oxidative and reductive reactions they act as sinks and sources of protons and they may help in the regulation of cytoplasmic pH.

1.6.4 Factors affecting the accumulation of polyols

The accumulation of polyols in filamentous fungi could be a generalised response to exposure to lowered water activities. However, *in vitro* studies have shown that the ratios of accumulated polyols are affected by the fungal species, the level of a_w in the external medium, and the type of compound used to reduce the external a_w (Hallsworth & Magan, 1994b). Adler *et al.* (1982) studied the polyol accumulation by *Aspergillus niger* and *Penicillium chrysogenum* and they suggested that microorganisms grown under water-stress imposed by salt preferentially accumulated glycerol while sugar rich media promote the accumulation of longer chain polyols. However, the prevailing

polyols accumulated in a fungus (at least in Deutoromycetes) appears to be the metabolically closest to the nutrient available in the medium. For example, *M. anisopliae* accumulated more glycerol when the growth medium was modified by glycerol than by glucose (Hallsworth & Magan, 1994b). However, both of the above studies (Adler *et al.*, 1982; Hallsworth & Magan, 1994b) showed a shift towards synthesis of lower molecular weight compounds such as erythritol and glycerol when the a_w was lowered progressively. The same trend was also observed for some other fungal species including BCAs (Kelly & Budd, 1991; Pascual *et al.*, 1999; Pascual *et al.*, 2000a).

The different polyol accumulation patterns under different environmental and chemical growth conditions can have important implications for the germinability of fungi under low environmental water availability conditions. The lower molecular weight polyols glycerol and erythritol can be more effective in osmotic adjustment than the higher molecular weight polyols since saturated solutions of glycerol and erythritol have a a_w of less than 0.914 at 25°C compared to 0.978 for saturated mannitol solution at the same temperature (Hallsworth & Magan, 1995). For example, conidia of *B. bassiana*, and *M. anisopliae* with increased levels of glycerol were associated with higher germination rates and more rapid germ tube growth *in vitro*, relative to those from controls at both high and low water availability conditions (Hallsworth & Magan, 1995). Additionally, conidia containing high concentrations of glycerol and erythritol may cause insect mortality more rapidly and at lower RH levels than propagules with low levels of these polyols (Hallsworth & Magan, 1994c). Further evidence of improved pathogenicity of a fungal BCA (mycopathogen in this case) when produced under low a_w levels comes from a study by Harman *et al.* (1991). They found that spores produced in liquid media modified with polyethylene glycol (PEG) were more effective than spores produced in a medium without osmoticums. However, no polyol determinations were carried out and therefore no correlation of the improved biocontrol with polyol accumulation could be made. The accumulation of polyols when fungi are produced under water-stress imposed by different solutes can be considered to physiologically manipulate endogenous reserves when producing propagules of entomopathogenic fungi.

Water-stress is not the only factor that can induce polyol accumulation in fungi. Other factors such as culture age (Hallsworth & Magan, 1996; Pascual *et al.*, 1999; 2000a; Frey & Magan, 2001), pH and temperature can also influence polyol accumulation in fungi (Hallsworth & Magan, 1996). However, in a study with the entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *Paecilomyces farinosus*, none of these three factors led to a remarkable accumulation of polyols (Hallsworth & Magan, 1996) when compared with reports when these fungi where osmotically stressed using different solutes (Hallsworth & Magan, 1994a, b). These factors must be considered when producing propagules of fungal BCAs so as to determine the optimum conditions for obtaining the best quality of propagules as well as yield. The effect of interaction between these factors on polyol accumulation should also be examined.

Most of the studies about polyol accumulation in fungal propagules have been done for aerial produced structures or for mycelial fragments produced in submerged fermentation. On the other hand, there is no information about the effect of different cultural environmental stress conditions on the accumulation of polyols in blastospores. This kind of fungal propagule formation presents a high interest as a commercial formulated product because its faster germination rate compared to aerial produced conidia may provide them with an ecological advantage in inciting disease in insect pests (Fargues *et al.*, 1994; Vega *et al.*, 1999). Additionally, blastospores are produced by submerged fermentation which is a very cheap and a consistent method of fungal fermentation. This work presents a comprehensive study of the effect of different environmental liquid cultural conditions on the accumulation of polyols in *M. anisopliae* blastospores.

1.6.5 Mechanisms of polyol accumulation in fungi

Two mechanisms of polyol accumulation have been proposed. The first one is through direct absorption by diffusion with polyols of low molecular weight being able to more readily permeate the membrane than polyols of higher molecular weight except mannitol which is unable to be absorbed by diffusion (Kiyosawa, 1991). The second mechanism suggests intracellular synthesis by internal or external carbohydrate reserves. The fact that polyols are synthesised intracellularly comes from studies where

the substrate a_w was lowered using a non-metabolisable compound such as KCl (Hallsworth & Magan, 1994a). Kelly & Budd (1991) showed that exogenous glucose was clearly the source of essentially all carbon for polyol synthesis under water-stress conditions. They also suggest that the mechanisms adjusting polyol pool composition under varying degrees of osmotic stress could be the regulation of enzyme synthesis since the response to polyol accumulation under osmotic stress was prevented by cycloheximide, an inhibitor of translation in eukaryotes. Studies that show response to polyol accumulation in the absence of an exogenous carbohydrate reserve that could elucidate whether internal reserves are utilised for polyol synthesis, are lacking.

1.7 METHODS OF MYCOINSECTICIDE PRODUCTION

Production of fungi as BCAs aims at the maximum possible number of high quality propagules. Both liquid and solid fermentation systems can be used to obtain a desirable quantity of biomass and both systems have advantages and disadvantages.

1.7.1 Solid substrate fermentation

Solid substrate fermentation can be a very cheap method of mass production of fungal BCAs since very high numbers of spores can be produced using cheap raw materials like agricultural products (heterogeneous cereals or by-products). Additionally, propagules produced on solid substrates tend to be more tolerant to desiccation and more stable as a dry preparation compared to spores produced under submerged liquid fermentation (Goettel & Roberts, 1992). An alternative to agricultural products are inert materials (e.g. vermiculite) impregnated with liquid medium which have been successfully used as substrates (Ooijkaas, 2000).

Problems faced in solid substrate fermentation are substrate sterilisation, gas exchange, temperature control, long fermentation times (sometimes more than 9 days), and product recovery. The method can sometimes be labour-intensive and uneconomical (Deshpande, 1999).

1.7.2 Liquid fermentation

Liquid fermentation as opposed to solid substrate fermentation has the advantages of being a controlled process, ease of product harvesting and short fermentation times (Deshpande, 1999). Although mycopathogenic fungi have commonly been reported to produce conidia in liquid cultures (Harman *et al.*, 1991; Pascual *et al.*, 1997; Frey & Magan, 2001), in most cases when grown in liquid culture mycelial fragments and blastospores are produced. The latter being produced by budding off from the hypha. There are reports, however, of formation of conidia in liquid culture by the entomopathogenic fungi *B. bassiana* and *Metarhizium flavoviride* (Thomas *et al.*, 1987; Jenkins & Prior, 1993).

The main limitation with liquid fermentation is that submerged spores are usually short-lived and do not survive adverse environmental conditions as well as aerially produced conidia (Lane *et al.*, 1991a; Muñoz *et al.*, 1995; Pascual *et al.*, 2000b; Frey & Magan, 2001). Submerged blastospores produced by the entomopathogenic fungus *M. flavoviride* are totally devoid of the extra warty outer layer observed in the case of aerial conidia and submerged conidia of the same fungus possess this layer but to a lesser extent compared to aerial conidia (Jenkins & Prior, 1993). The same observations have been reported for the mycopathogenic fungus *Trichoderma harzianum* (Muñoz *et al.*, 1995). Additionally, aerially produced spores of *T. harzianum* were highly hydrophobic compared to submerged ones (Muñoz *et al.*, 1995). These properties make aerial conidia less vulnerable to environmental conditions. However, there is the potential to improve the stability of submerged produced spores. Methods reported include growing the fungus at reduced a_w (Jin *et al.*, 1991), by culturing the fungus under different carbon and nitrogen ratios (Lane *et al.*, 1991a), modification of the nutritional status of the liquid culture, especially as far as nitrogen source is concerned (Jackson *et al.*, 1997; 2003), or by storage in appropriate liquids such as 10% hydroxyethyl starch (Kleespies & Zimmermann, 1994). Liquid fermentation provides a system for rapid and cheap fungal propagule production.

1.8 FORMULATION OF ENTOMOGENOUS FUNGAL BIOLOGICAL CONTROL AGENTS

Formulation can improve certain characteristics of entomogenous fungal BCAs and is the only way to make such agents commercially viable. Jones & Burges (1997) list four main functions of formulation: 1) to stabilise the microorganism during production, distribution and storage; 2) to improve handling and application of the product; 3) to protect the agent from harmful environmental factors at the target site and 4) to improve activity of the agent at the target site by increasing its activity, reproduction, contact and interaction with the target pest. Although formulation is important both from the point of application and storage, this work mainly considers the improved storage stability. Jones *et al.* (1997) reviewed the aspects of fungal biocontrol agent formulation from an application perspective.

A fungal BCA must retain its stability for at least one year to be considered for commercial development (Rodham *et al.*, 1999). Stability for 18-24 months at room temperature (25°C) is required to increase market competitiveness, but it has remained an elusive goal (Wraight *et al.*, 2001). However, considerable progress is being made. There are several ways by which the stability of a product can be improved and include a) selection of fungal species or strain, b) appropriate production conditions, c) post-production processing and d) addition of certain additives in the formulation product.

1.8.1 Selection of fungal species or strains

It is possible to select species or strains of an entomopathogenic fungus that are more tolerant to external factors and survive storage better. For example, Kleespies & Zimmermann (1994) tested the viability and virulence of blastospores of *M. anisopliae* after storage in various liquids and at different temperatures and found that best survival depended on the fungal strain. As mentioned previously, *M. flavoviride* will produce conidia in liquid culture (Jenkins & Prior, 1993) which are more robust when compared to blastospores. Therefore, by selection of the appropriate fungal species production of

propagules with the desirable properties of aerial conidia can be obtained while exploiting the advantages of the short fermentation time.

1.8.2 Appropriate production conditions

Conditions under which a fungus is produced can affect storage stability. Several studies have examined this possibility (Jin *et al.*, 1991; Lane *et al.*, 1991a; Hallsworth & Magan 1994c; Jin *et al.*, 1996; Cliquet & Jackson, 1999; Jackson *et al.*, 2003). These studies mainly considered the careful manipulation of C:N ratios, careful selection of nitrogen sources and production at low water availability using appropriate solutes. For example, by growing *B. bassiana* in nitrogen and carbon-limited liquid culture Lane *et al.* (1991a) found that blastospores harvested from the stationary phase from nitrogen-limited cultures survived longer than those from carbon-limited cultures. They attributed this to the fact that blastospores from nitrogen-limited cultures contained higher levels of total carbohydrate, glycogen and total lipid. Improved stability of a fungal product by production of propagules at low a_w relies on potential accumulation of trehalose in the produced cells (Jin *et al.*, 1991). Trehalose is capable of stabilising membranes during dehydration (Crowe *et al.*, 1984) and confers protection of membranes during a drying process and subsequent enhanced viability and persistence of dried cells. Selection of appropriate nitrogen and/or carbon sources during fermentation of a fungal biocontrol agent can also influence its tolerance to desiccation and stability as a product (Cliquet & Jackson, 1999; Jackson *et al.*, 2003).

1.8.3 Post-production processing

The methods used to process fungal biocontrol agents after their production can influence their storage stability. The majority of formulations exhibiting sufficient shelf-life require biocontrol propagules to be in a dry state because in this way all metabolic processes are dramatically slowed down. Methods of drying include spray-drying, freeze-drying, air-drying and fluidised bed drying. Care must be taken however, during drying processes so that key biological structures such as membranes, proteins and nucleic acids retain their function. It is generally thought that loss of viability during dehydration occurs due to membrane damage. A number of polyhydroxy compounds such as trehalose, sugars and polyols are believed to protect membranes during

dehydration (Crowe *et al.*, 1984; Carpenter & Crowe, 1988; Hoekstra *et al.*, 1997). Therefore such compounds can be used as protectants during the drying of spores. The spray-drying of *Beauveria* spp. without the use of protectants caused complete loss of spore viability (Feng *et al.*, 1994), but 20 species of yeasts, which are reported to be sensitive to freeze-drying and liquid-drying, were successfully dried using a solution of adanitol, meso-inositol, honey, Na-$_L$-glutamate and trehalose (Malik & Hoffmann, 1993). Stephan & Zimmermann (1998) also found that when *M. anisopliae* blastospores were air-dried using skimmed milk powder in combination with sugar-beet syrup, they were as virulent to *Locusta migratoria* as fresh blastospores.

Clays have also been reported to enhance spore survival, possibly due to their desiccation properties (Wraight *et al.*, 2001), but Moore & Higgins (1997) found no improvement in the stability of *M. flavoviride* conidia when they had been dried to 7% moisture content and formulated with and without various clays. Reducing the moisture content of *M. flavoviride* conidia by drying under low humidity resulted in increased storage longevity (Hong *et al.*, 1998).

The composition of the storage atmosphere is another important factor in fungal stability and storage under nitrogen or enriched CO_2 atmospheres and also under vacuum are known to enhance short-term stability of aerial conidia (Wraight *et al.*, 2001).

Preservation in oil or in other appropriate liquids is another promising way of improving fungal stability. Moore *et al.* (1995) for example, used a mineral and two vegetable oils to store dried conidia in silica gel and undried conidia of *M. flavoviride* and found that although during the first 26 weeks of storage there was no significant difference in the survival of the dried conidia, after this time and for up to 88 weeks the mineral oil was superior to the vegetable oils. However, the most important factor that determined long term stability was the drying with silica gel since viability of the undried spores dropped below 40% after 9 and 32 weeks at 17 and 8°C respectively. Dried spores germinated at over 60 and 80% after 127 weeks in storage at 17 and 8 °C respectively. Temperature played an important role in survival during storage. Interestingly, blastospores of *M.*

anisopliae (strain 57) stored for 18 weeks at 4°C and 100% PEG had better virulence on third instars of the African migratory locust *L. migratoria* compared to fresh aerially produced conidia (Kleespies & Zimmermann, 1994). The same was observed with a different strain of the same fungus after storage for 22 weeks at 4°C in 10% glycerol solution but against adults of *L. migratoria*.

Most studies about formulation of fungal biocontrol agents have considered the steps during production and drying processing of the desired product. One step that has not been given sufficient attention is the handling of the final fungal product during harvest. Production of fungal biocontrol agents employ some kind of medium (liquid or solid) amendment by choosing appropriate nutritional components of the medium, or adjusting its physicochemical properties i.e. changing the a_w, pH, viscosity. All these modifications may result in accumulation of different compounds in the produced cells, such as polyols, sugars, lipids which could be beneficial for subsequent stability and efficacy of the product. It is important therefore, to preserve such compounds and prevent any loss of them throughout processing of a fungal biocontrol agent.

Many studies have used immersion in water as a method of harvesting the produced fungal propagules (Jin *et al.*, 1991; Fargues *et al.*, 1994; Costa *et al.*, 2000; Fargues *et al.*, 2001), a procedure which may result in leakage of intracellular compounds out of the cell (Luard, 1982b; Kelly & Budd, 1991; Pascual *et al.*, 1999). When cells are grown in modified medium (especially liquid medium) they adjust their intracellular water potential according to the water potential of the external environment. Subsequent immersion in water results in hypo-osmotic shock and possible loss of endogenous compounds. Such an effect has been reported previously for the obligate xerophilic fungus *Chrysosporium fastidium* (Luard, 1982b). It is now widely accepted that different sugars protect fungal membranes during dehydration, and several studies have tested the effect of immersion of fungal propagules in sugar solutions before a drying process (Cliquet & Jackson, 1999; Montazeri & Greaves, 2002), with successful outcomes. However, there has not been any study on the effect of accurate harvesting and washing of fungal propagules in iso-tonic solutions on subsequent viability and

stability of a fungal biocontrol agent. The present study has given particular attention to this neglected step during the production of modified propagules of *M. anisopliae*.

1.8.4 Formulation additives

Additives in a fungal BCA formulation can enhance stability by enhancing storage properties. Fungal BCAs are mainly stored in their resting stage form, their spores, and additives that prevent premature growth may be needed (Jones & Burges, 1997). Other additives, such as silica, which prevents caking of the product, may also be necessary. Some additives are also used to improve efficacy of the fungal BCA after application. Good information concerning this aspect can be found in Jones & Burges (1997); Jones *et al.* (1997) and Wraight *et al.* (2001).

1.9 RESTRICTIONS ON THE USE OF ENTOMOPATHOGENIC FUNGI

Apart from the factors influencing the efficacy of fungal BCAs which can restrict the use of entomopathogenic fungi, there are other obstacles which have to be overcome for the successful commercial development of a fungal biocontrol agent. These mainly include the costs of development and registration, education of the farmers on the appropriate usage of the fungal biopesticides, and poor shelf-life.

1.9.1 Mass production and ecological fitness of the inoculum

One of the most important features for a successful fungal BCA is the ease with which it can be produced. Fungal BCAs are usually host and environment specific and thus aimed at small niche markets. Additionally they have to compete with the broad-spectrum insecticides, the overall development cost of which is often lower. Therefore their economic feasibility will be greatly dependent on cost-effective mass production.

However, in an effort to make fungal biocontrol agents economically feasible, most research has focused on optimising the quantity of infectious propagules, largely neglecting the quality of inocula. This has often led to the production of inocula with

low efficiency in natural conditions and subsequent failure of its commercial development. Fungal BCAs will encounter fluctuating abiotic factors, such as water availability, temperature, length of dew periods, microclimate and rainfall events, when applied in the field. A successful biocontrol agent will benefit from improved ecological fitness and the ability to tolerate such factors and retain its effectiveness (Magan, 2001). Therefore research must interdependently consider cost-effective mass production and ecological fitness of produced inocula. This study concentrates on these aspects.

1.9.2 Registration of fungal biological control agents

Fungal biocontrol agents must be shown to be safe to humans and other non-target organisms before being commercially developed. Fungi contain many species that are pathogens themselves or may become so when introduced into an environment where they never existed before. A good example of this is the potato blight in Europe during the 19th century caused by the plant pathogen *Phytopthora infestants* (Goettel *et al.*, 2001). In the case of entomopathogenic fungi problems could mainly arise from their potential interference with beneficial insects, such as predators and parasitoids, disturbing the equilibrium between these and pests and causing pest resurgence not known before. This concern about releasing non-indigenous microorganisms reflects the problems associated with registration requirements in different countries. Careful screening, therefore, concerning their potential effect on non-target organinsms including vertebrates and humans is required. However, Moore & Prior (1993) argued that although natural epizootics and those induced by mycoinsecticides have resulted in enormous levels of conidia entering ecosystems, no marked environmental problems have been noted and there have been no reports of human or other vertebrate infections. No candidate fungal biocontrol agent has been reported to cause an epizootic in honey bees (Cook *et al.*, 1996) even though some have shown pathogenicity to honey bees in laboratory studies (Butt *et al.*, 1994).

Many fungi secrete toxic metabolites that might be harmful to plants, beneficial invertebrates and vertebrates (Goettel *et al.*, 2001). Therefore toxicological studies are also required in addition to screening for potential hazardous effect on non-target organisms before their commercial development. This entails high costs which have

often stifled successful commercialisation of fungal BCAs. The costly and controversial procedure of risk assessment has resulted in commercial production of some fungal BCAs which are registered as some form of 'plant growth promoter', 'soil conditioner', 'plant strengthener', or 'wound protectant' rather than as BCAs (Whipps & Lumsden, 2001). By not claiming biopesticide activity there is no requirement for registration but safety cannot be assured without toxicological studies and consistent beneficial effects are not always seen without efficacy data. However, Vey *et al.* (2001) claim that there are neither documented accounts of toxin levels rising as a result of artificially induced or natural epizootics, nor reports of fungal metabolites produced by BCA entering the food chain.

1.9.3 Persuading farmers to use mycoinsecticides

Another constraint on the use of mycoinsecticides comes from the unwillingness of farmers to use them for a number of reasons. Mycoisecticides may need 2-3 days after application to have an effect and they may not lead to complete extermination of the target pest. Additionally, they may be inconsistent in their efficacy. All these features are seen with scepticism by farmers who are used to a rapid, total extermination of the target pest with the use of chemicals. Mycoinsecticides often need more specific environmental requirements and better timing than chemicals to be effective. However, with complete and accurate instructions, or short training sessions, the effective use of microbial insecticides can become feasible. Effective use of fungal biocontrol agents can be also achieved in integrated control management where BCA are used in combination with some spray chemicals.

1.9.4 Poor shelf-life

Shelf-life is a very important factor that influences the economic feasibility of a mycoinsecticide. Entomopathogenic fungi may lose their viability in a matter of weeks or store well only when refrigerated. This adds cost to the product and makes it more difficult to be commercially viable. However, good progress in improving mycoinsecticide stability is being made and the ways that this can be achieved have already been discussed in Section 1.8. Additionally, drying of entomopathogenic

conidia under low humidity (58% R.H.) and storage at equilibrium R.H. can result in good longevity (>80% viability after 120 days in storage) (Hong *et al.*, 1999).

1.10 OBJECTIVES

In this work the entomopathogenic fungus *M. anisopliae* was used as the model microorganism to study the effect of modification of solid and liquid cultural conditions on the production and quality of produced propagules. The aim of this study was to determine optimum cultural conditions under which high numbers of propagules with enhanced germination efficiency under low water availability conditions are produced in the shortest possible fermentation time. Additionally, resistance of propagules to drying and subsequent stability in storage was also an important consideration. To this end the following steps were followed:

A. SOLID FERMENTATION

- Production of conidia in solid substrate fermentation using 2 different types of substrate (bulgar wheat and millet grains) modified to different a_w levels with and without addition of a carbohydrate source
- Determination of the endogenous concentration of selected compatible solutes in conidia harvested at different times after incubation under different solid substrate cultural conditions
- Evaluation of germination efficiency under conditions of water-stress
- Selection of the best conidial treatment for subsequent drying processing and storage stability studies

B. LIQUID FERMENTATION

- Production of blastospores under different cultural regimes (a_w, nitrogen status, pH)
- Determination of the endogenous concentration of selected compatible solutes and total protein content in blastospores harvested at different times after incubation under different cultural regimes
- Evaluation of germination efficiency under conditions of water-stress

- Effect of osmoprotection of blastospores produced under water-stress on endogenous reserve retention and germination effeciency
- Selection of the best blastospore treatments for subsequent quality studies

C. EFFECT OF OSMOPROTECTION OF MODIFIED BLASTOSPORES ON QUALITY CHARACTERISTICS

- Effect of osmoprotection and type of osmoprotectant on extracellular protease and chitinase activity
- Effect of osmoprotection and type of osmoprotectant on extracellular production of destruxin A
- Comparison between the best blastospore treatments and the best conidial treatment in terms of enzyme activity and destruxin A production

D. EFFECT OF OSMOPROTECTION OF MODIFIED BLASTOSPORES ON STABILITY

- Effect of osmoprotection and type of osmoprotectant on storage stability as wet pastes
- Effect of osmoprotection and type of osmoprotectant on freeze-drying tolerance and subsequent storage stability
- Comparison between the best blastospore treatments and the best conidial treatment in terms of stability as freeze-dried spores

E. BIOASSAY EVALUATION OF POTENCY OF MODIFIED BLASTOSPORES IN PATHOGENICITY IN APHIDS AND THE EFFECT OF OSMOPROTECTION

- Evaluation of the potency of 2 of the characterised best blastospores in pathogenicity assays with aphids
- Effect of osmoprotection on pathogenicity of modified blastospores
- Comparison between modified blastospore treatments and the best conidial treatment

Figure 1.2 summarises the components of the project presented in this thesis. The work is presented as a series of Chapters (2-6) with integrated materials and methods, results and discussion.

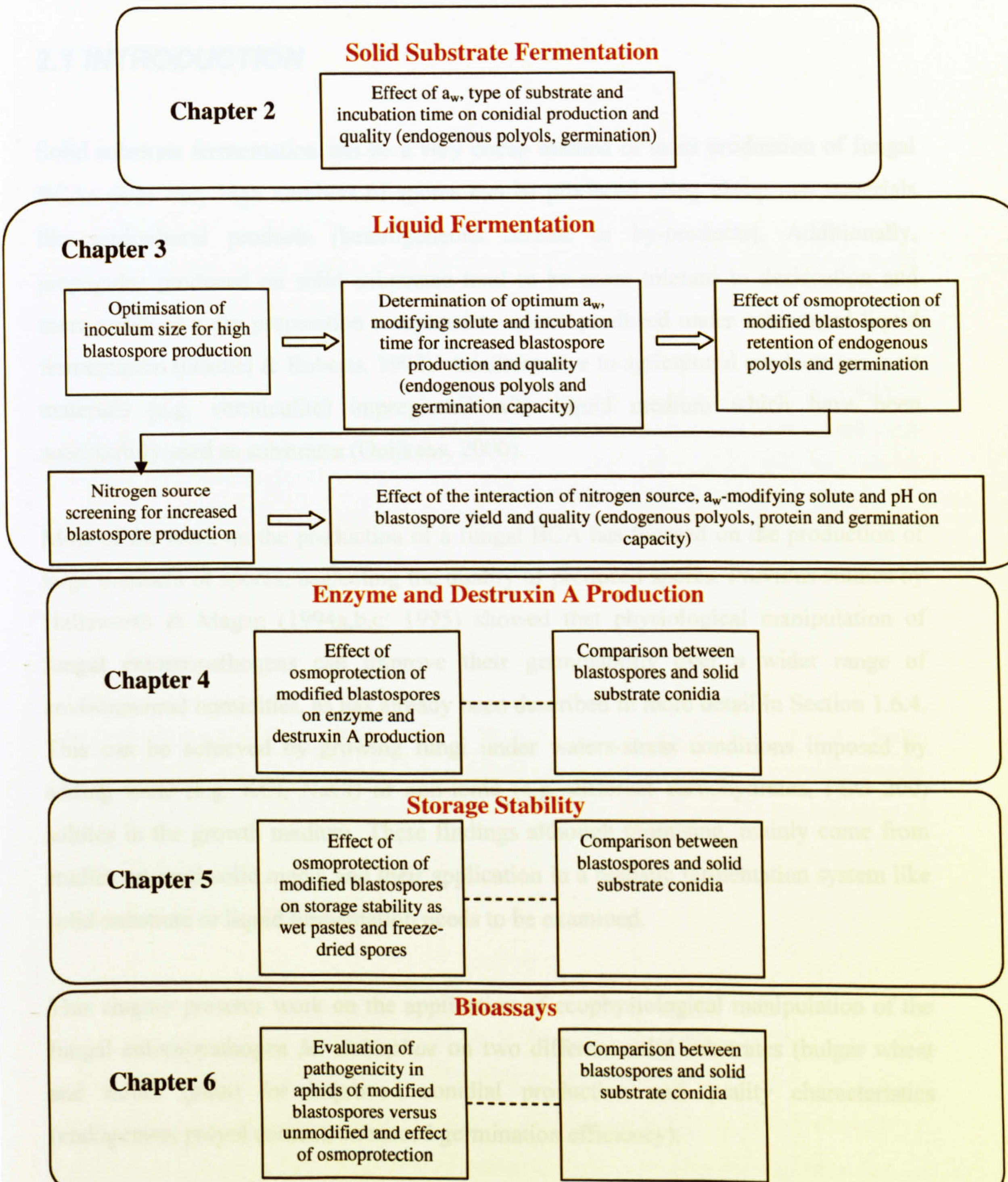


Figure 1.2. Flow diagram of the experimental work carried out in this thesis. Dotted lines represent experimental interconnectivity.

Chapter 2 SOLID SUBSTRATE FERMENTATION

2.1 INTRODUCTION

Solid substrate fermentation can be a very cheap method of mass production of fungal BCAs since very high numbers of spores can be produced using cheap raw materials like agricultural products (heterogeneous cereals or by-products). Additionally, propagules produced on solid substrates tend to be more tolerant to desiccation and more stable as a dry preparation compared to spores produced under submerged liquid fermentation (Goettel & Roberts, 1992). An alternative to agricultural products are inert materials (e.g. vermiculite) impregnated with liquid medium which have been successfully used as substrates (Ooijkaas, 2000).

Most of the work on the production of a fungal BCA has focused on the production of large numbers of spores, neglecting the quality of produced spores. Previous studies by Hallsworth & Magan (1994a,b,c; 1995) showed that physiological manipulation of fungal entomopathogens can improve their germinability over a wider range of environmental humidities, as has already been described in more detail in Section 1.6.4. This can be achieved by growing fungi under waters-stress conditions imposed by adding ionic (e.g. KCl, NaCl) or non-ionic (e.g. different carbohydrates, PEG 200) solutes in the growth medium. These findings although promising, mainly come from studies on semi-solid media and their application in a realistic fermentation system like solid-substrate or liquid fermentation needs to be examined.

This chapter presents work on the application of ecophysiological manipulation of the fungal entomopathogen *M. anisopliae* on two different solid substrates (bulgar wheat and millet grain) for improved conidial production and quality characteristics (endogenous polyol content, enhanced germination efficiency).

2.2 MATERIALS AND METHODS

2.2.1 Fungal species

Cultures of *M. anisopliae* (416.96) were provided by Warwick Horticulture Research International (HRI), Wellesbourne. The cultures were stored in sterile 15% glycerol solutions at -80°C. The fungus was grown on malt extract agar (MEA) (Merck, U.K.) at 25°C for 10-14 days before it was used in any of the experiments.

2.2.2 Construction of moisture sorption isotherms

Bulgar wheat and millet grains were the two heterogeneous media used in the solid substrate fermentation experiments. In order to modify the a_w of the solid media, moisture sorption isotherms of the grains modified with water alone or water/glycerol solutions, were constructed following a_w measurements of modified grain samples. Where water alone was used, volumes of distilled water over a range of 0-7.0 ml were added to 10 g subsample of grain (bulgar wheat or millet) in Universal bottles. These were autoclaved for 30 min at 121°C and then left to equilibrate for 2 days at 4°C. After equilibration, a_w of the samples was measured using a Aqualab water activity meter (model 3 TE, Labcell Ltd., Basingstoke, Hants, U.K.) and the amount of water needed to saturate each type of grain was noted. Five grams of each sample were also weighed and oven dried at 106°C overnight (18 h). After drying, the samples were re-weighed to determine moisture content values. Where water/glycerol solutions were used to modify the solid media, the same procedure was followed, except that water/glycerol solutions with glycerol concentration ranging from 0 - 41.4 g 100 ml^{-1} of water were added to 10 g subsample of grain. The volume of water/glycerol solutions was the equivalent of that needed to saturate the corresponding medium with water.

2.2.3 Water activity modification

The two types of grain were modified by water alone to saturation (0.99 $a_w \equiv$ 99% environmental relative humidity; E.R.H.), 0.98 (± 0.003) and 0.96 (±0.003) a_w levels or by water/glycerol solution to 0.98 (± 0.003) and 0.96 (± 0.003) a_w levels according to the moisture sorption isotherms. Modification of a substrate to each a_w was done in separate 500 ml Duran bottles (up to 250 g of fresh grain per bottle) and left to

equilibrate for 2 days at 4°C. When millet grain was used, samples were autoclaved for 30 min before they were left to equilibrate, because preliminary experiments showed that absorption by this grain was poor if left to equilibrate without autoclaving.

2.2.4 Experimental design

Vented jars each containing 20 g of modified grain of the appropriate a_w level were autoclaved for 50 min at 121°C on two consecutive days. After that, jars containing modified grain of the same a_w were placed together in closed sandwich boxes where E.R.H. was maintained at that corresponding to the enclosed substrate by water/glycerol solutions prepared according to Table 2.1 and left for 24 h at 25°C to equilibrate.

2.2.5 Inoculation, incubation and harvest procedure

Each jar received 0.25 ml of inoculum prepared by suspending *M. anisopliae* conidia grown on MEA for 10-14 days in sterile water + 0.1% (w/v) Tween 80 (Sigma, U.K.) solutions. The small inoculum of 0.25 ml was used to prevent substantially modifying the a_w of the treatments.

After inoculation, jars containing modified grain of the same a_w were placed together again in their corresponding enclosed sandwich boxes and incubated at 25°C. In the case of bulgar wheat the experiment was run for 15 days with three replicates per treatment destructively taken after 5, 10 and 15 days to assess spore production and germinability. Because spore germinability was found to be reduced considerably after 15 days incubation, the experiment with millet was subsequently run for 10 days with spores harvested after 5 and 10 days. For spore harvesting the substrate was washed with 40 ml of sterile distilled water + 0.01% (w/v) Tween 80. The resultant liquid was filtered through one layer of Lens cleaning tissue (Whatman 105), and a sample was taken to count the spore numbers using a haemocytometer. The number of spores was referred back to the initial amount of substrate in a jar. The filtrate was then centrifuged at 3000 revolutions per minute (r.p.m) for 10 min after which the supernatant was discarded. Some spores from the pellet were used for germinability studies and the rest were subjected to High Performance Liquid Chromatography (HPLC) for sugar and sugar alcohol (polyol) analysis of spore contents.

Table 2.1. Concentrations of glycerol used to modify water at different a_w level (Dallyn & Fox, 1980).

Water activity	g glycerol / 100 ml H_2O
0.995	2.76
0.991	4.61
0.982	9.2
0.971	14.72
0.964	18.4
0.955	23.00
0.937	32.2
0.928	36.8
0.919	41.4

2.2.6 Germinability tests

Spores from the pellet formed as described above were suspended in sterile water/0.01% (w/v) Tween 80 solutions to give spore suspensions of $5x10^6$ spores ml^{-1}. Unmodified germination media were prepared using 2% technical agar No 3 (Oxoid) and 0.1% malt extract (Lab M) whereas stressed media were prepared using 2% technical agar No 3, 300 g of PEG 200 l^{-1} (Sigma, U.K.) and 0.1% malt extract of the final volume. When PEG 200 was added in the germination media, that was done prior to the addition of technical agar and malt extract, both of which were added after measurement of final volumes of PEG 200 solutions. The resultant a_w levels of the media were 0.998 and 0.963 respectively. Germination media were inoculated with 0.1 ml spore suspension from a treatment which was spread plated over the surface using a glass spreader. Petri plates of the same a_w were sealed in a polyethylene bag and incubated at 25°C. Fourteen hours after incubation three agar plugs from each replicate of the 0.998 a_w media were removed and stained with lactophenol cotton blue to halt further germination and help visualise germinating spores and germ tubes. The same procedure was used with the 0.963 a_w media, but 36 h after incubation. The percentage germination and germ tube lengths were assessed under the light microscope. Conidia with germ tubes as long as their length were considered to have germinated. To estimate the percentage germination, a total of 300 conidia were examined from each treatment

(100 per replicate), and 25 germinated conidia from each replicate plate were randomly chosen for measurement of germ tube length.

2.2.7 Polyol and sugar extraction procedure

Conidia (20-50 mg) were mixed with 1 ml HPLC grade water (Sigma, U.K.) in 2 ml Eppendorf tubes before being sonicated (Soniprep 150, Fisons) for 2 min at 28 μm amplitude. The samples were then boiled in a water bath for 5.5 min for optimum extraction, and allowed to cool (Hallsworth & Magan, 1997). As samples were ultimately to be subjected to HPLC analysis, it was necessary to adjust them in accordance with the mobile phase used. For this reason, 667 μl of acetonitrile (Sigma, U.K.) were added to each sample. The samples were shaken and centrifuged at 13000 r.p.m. for 15 min. After that they were filtered through 0.2 μm hydrophilic filters into HPLC glass vials. These vials were used for polyol and sugar analysis.

2.2.8 High performance liquid chromatography analysis

Solutes extracted from *M. anisoliae* spores were analysed using a Gilson HPLC (715) system. Following injection of 50 μl of sample, solutes were separated on a Ca^{2+} column (Hamilton HC-75) specially designed for sugar and polyol separation. This was connected to a Refractive Index Detector (R132 Anachem). The mobile phase consisted of 40:60 acetonitrile:HPLC grade water and before it was used it was sonicated for 10 min, and de-gassed with helium. Five standard sugar and polyol solutions of between 50 and 800 ppm were injected onto the column immediately before samples in order to calibrate the system. Quantification of solutes was carried out by peak area integration in relation to calibration curves (Gilson software). The system output (ppm) values for solutes was converted to mg solute g^{-1} sample, with the following equation:

$A \times (B/C)$ Where A = ppm of sugar or polyol analysed; B = 1.67 and is a conversion constant; C = fresh weight (mg) of spores in a sample.

2.2.9 Statistical analysis

Experiments were repeated 3 times and a representative set of data was selected for presentation and analysis. Results, where appropriate, were subjected to analysis of variance (ANOVA), or standard errors of the means were calculated. When significant differences were observed, means were compared by least significant difference (LSD) testing at $P = 0.05$. Data on percentage (%) germinability were logit transformed before statistical analysis and then back transformed for presentation. All data analysis was carried out on GenStat 5th edition Release 3 (Genstat 5 Committee, 1993). Where LSD values or bars are not presented in Figures, they can be found in Appendix I.

2.3 RESULTS

Before the solid substrate experiments were carried out, moisture sorption isotherms were developed for the substrates used. This enabled accurate modifications of a_w to be made.

2.3.1 Moisture isotherms of bulgar wheat modified with water alone or water/glycerol solutions

The relationship between the amount of added water (ml) and resultant a_w of fresh bulgar wheat is shown in Figure 2.1. Bulgar wheat was saturated when between 6-7 ml of water were added to 10 g subsamples. Figure 2.2 shows the relationship between bulgar wheat moisture content and a_w of the samples modified with water alone. The lowest data points in both Figure 2.1 and Figure 2.2 represent the sample where no water had been added. When the necessary volume of water to saturate 10 g of bulgar wheat was identified (6 ml), water/glycerol solutions of this volume with a range of glycerol concentration from 0 - 41.4 g/100 ml of water were added to different samples of 10 g of bulgar wheat. The relationship between added water/glycerol solutions and a_w for bulgar wheat is shown in Figure 2.3.

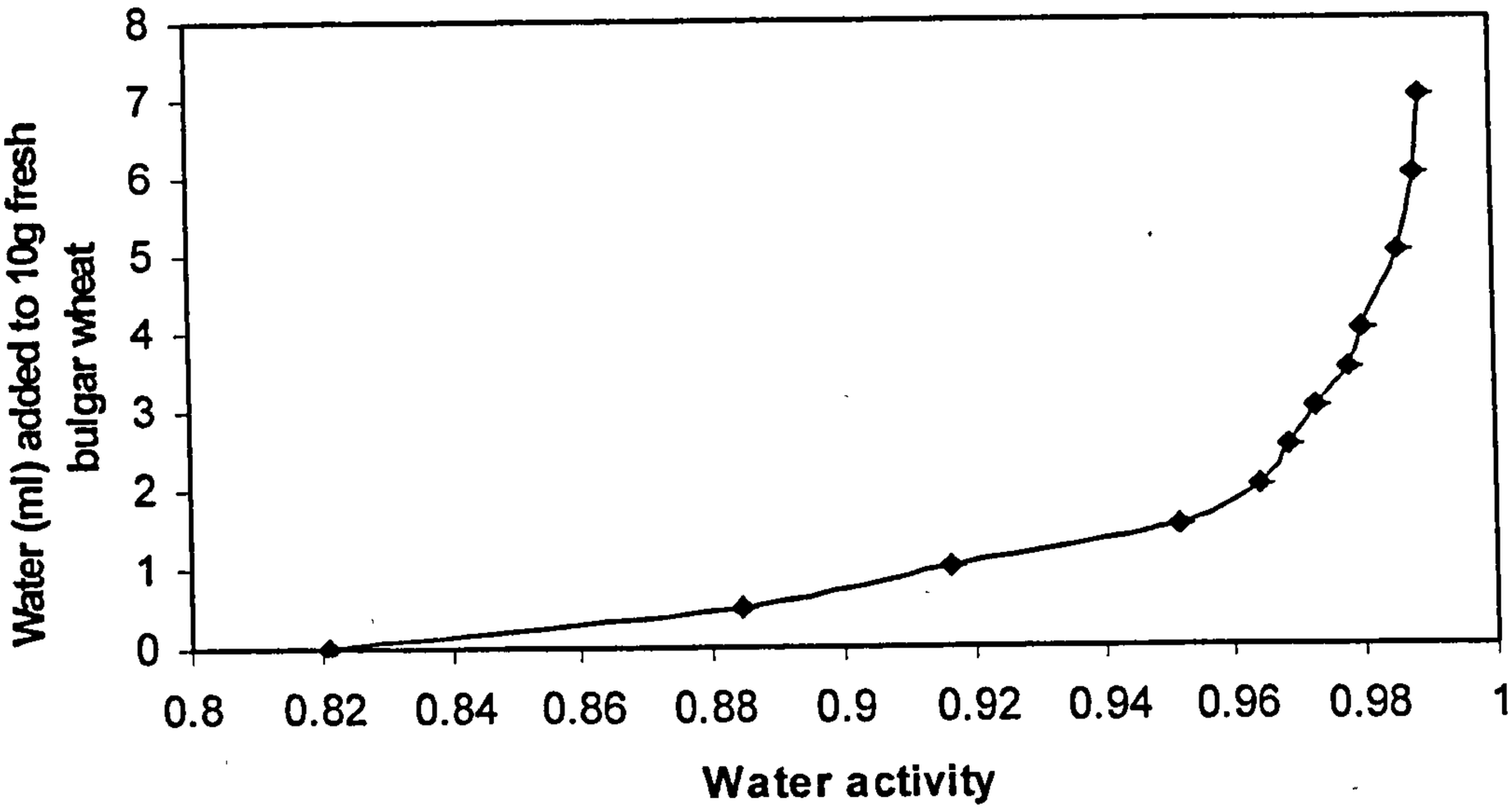


Figure 2.1. Water activity of 10 g fresh bulgar wheat after being soaked in various volumes of water for 48 h, (n=3). In all cases, the standard error of the means was smaller than the data points.

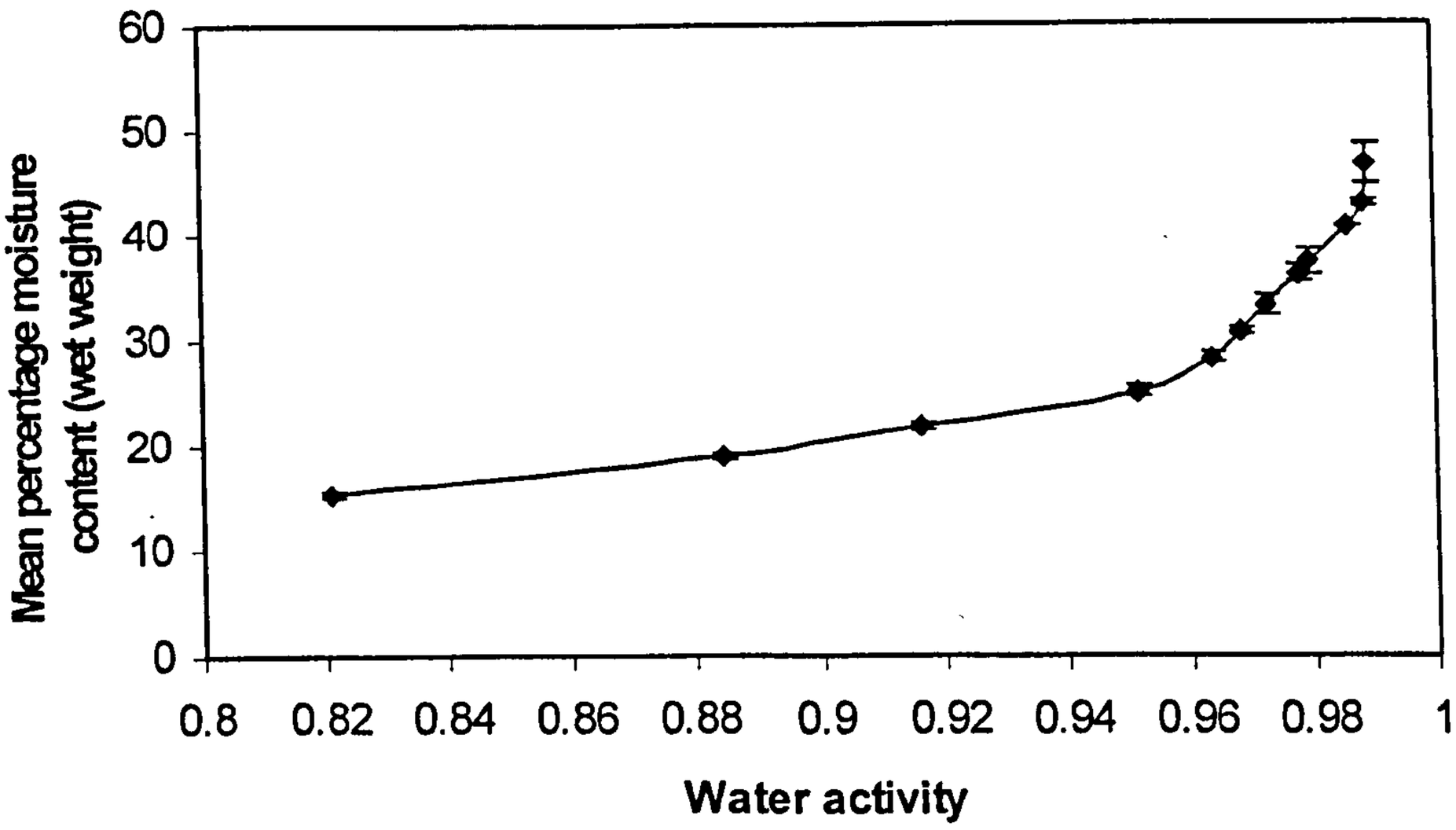


Figure 2.2. Relationship between percentage moisture content and a_w of 10 g fresh bulgar wheat modified by addition of different volumes of water. The moisture contents were determined by drying the modified samples at 106°C for 18 h. Bars where present, represent standard error of the means (n=3). In cases with no bars, standard error was smaller than the data points.

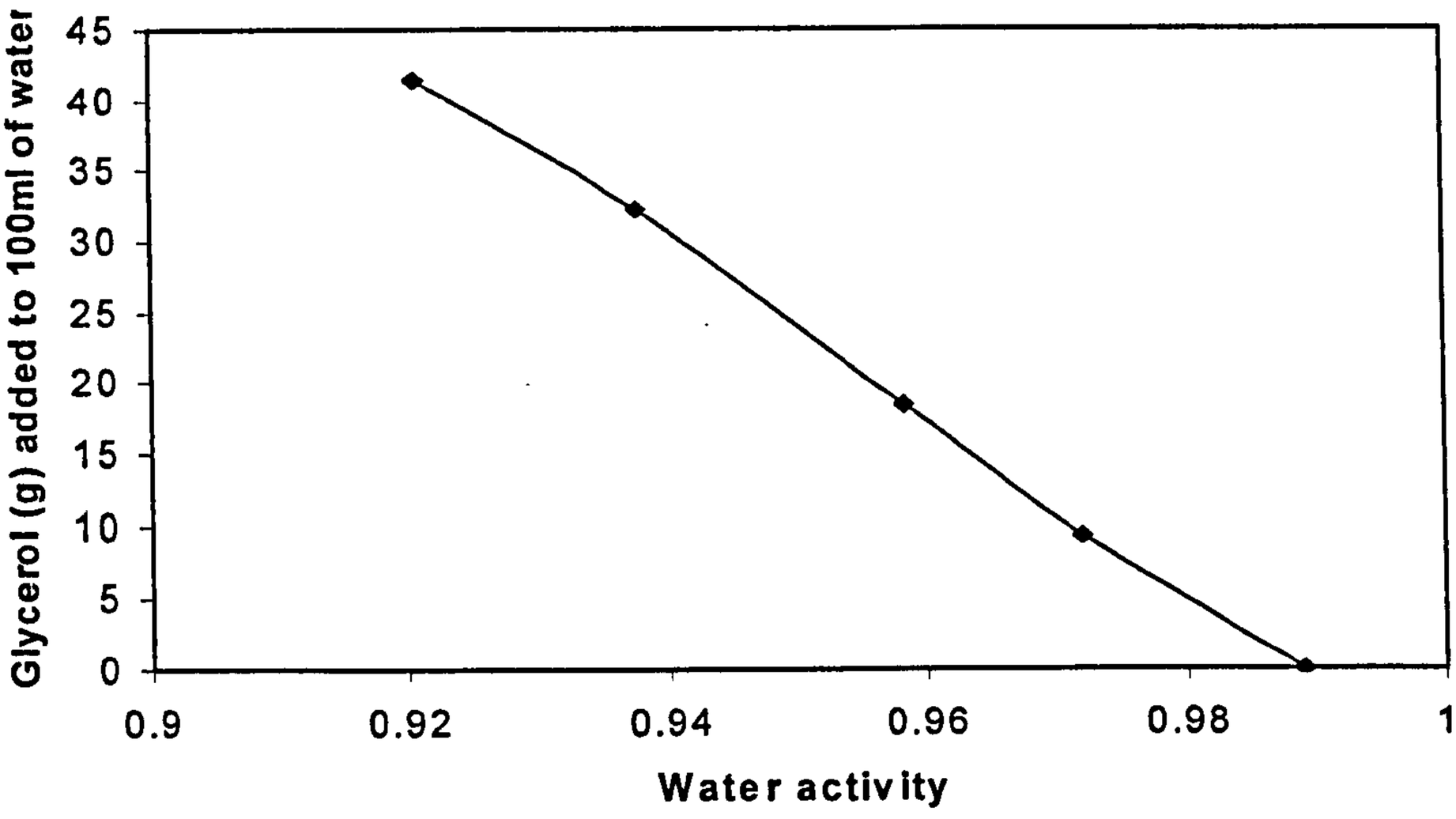


Figure 2.3. Water activity of 10 g fresh bulgar wheat after soaking in 6 ml of glycerol/water solutions with a range of glycerol concentration from 0 - 41.4 g/100 ml of water for 48 h (n=3). In all cases, the standard error of the means was smaller than the data points.

2.3.2 Moisture isotherms of millet modified with water alone or water/glycerol solutions

Figure 2.4 shows the relationship between the amount of added water (ml) to 10 g of millet and the resultant a_w. Ten g subsamples became saturated when 5-6 ml of water were added. The relationship between millet moisture content and a_w of the samples modified with water alone is shown in Figure 2.5. The lowest data points in both Figure 2.4 and Figure 2.5 represent the sample where no water had been added. The curve showing the relationship between water/glycerol solutions of different glycerol concentrations and resultant a_w of 10 g millet subsamples was constructed following the same procedure as in the case of bulgar wheat but using 5 ml solutions, and is shown in Figure 2.6.

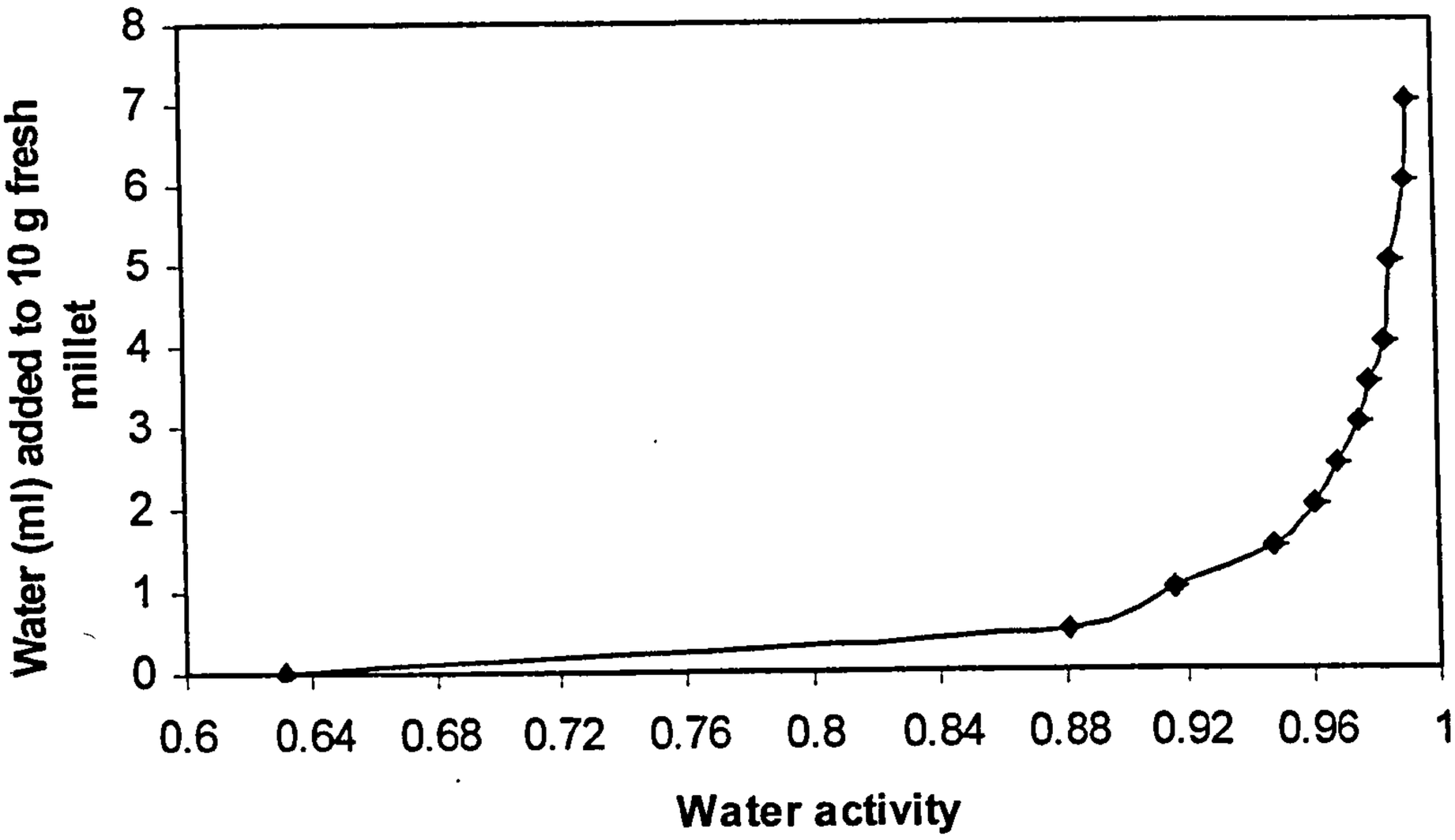


Figure 2.4. Water activity of 10 g fresh millet after soaking in various volumes of water, autoclaved for 30 min and then left for 48 h to equilibrate, (n=3). In all cases, the standard error of the means was smaller than the data points.

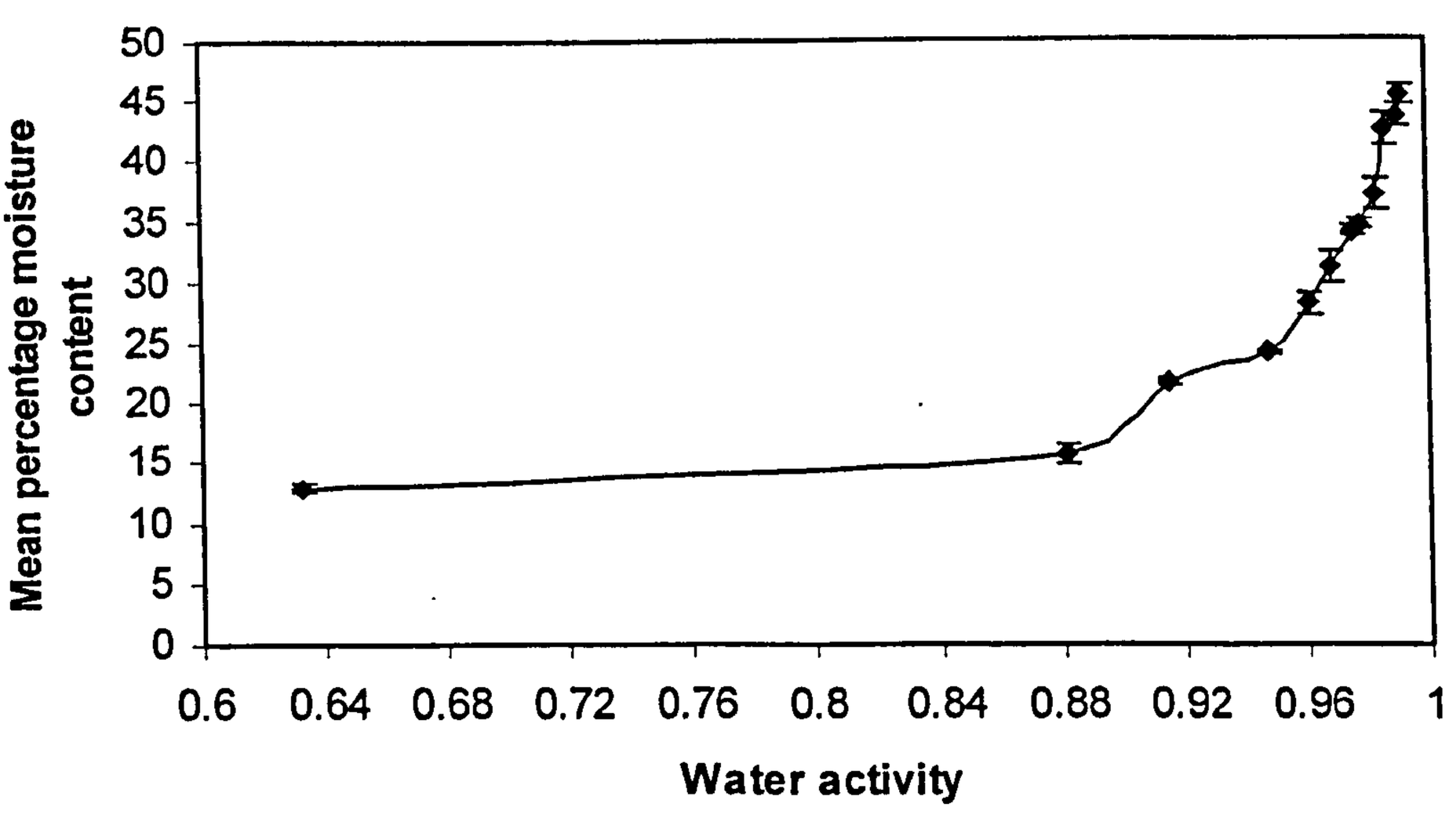


Figure 2.5. Relationship between percentage moisture content and a_w of 10 g fresh millet modified with different volumes of water. The moisture contents were determined by drying the modified samples at 106°C for 18 h. Bars where present, represent standard error of the means (n=3). In cases with no bars, standard error was smaller than the data points.

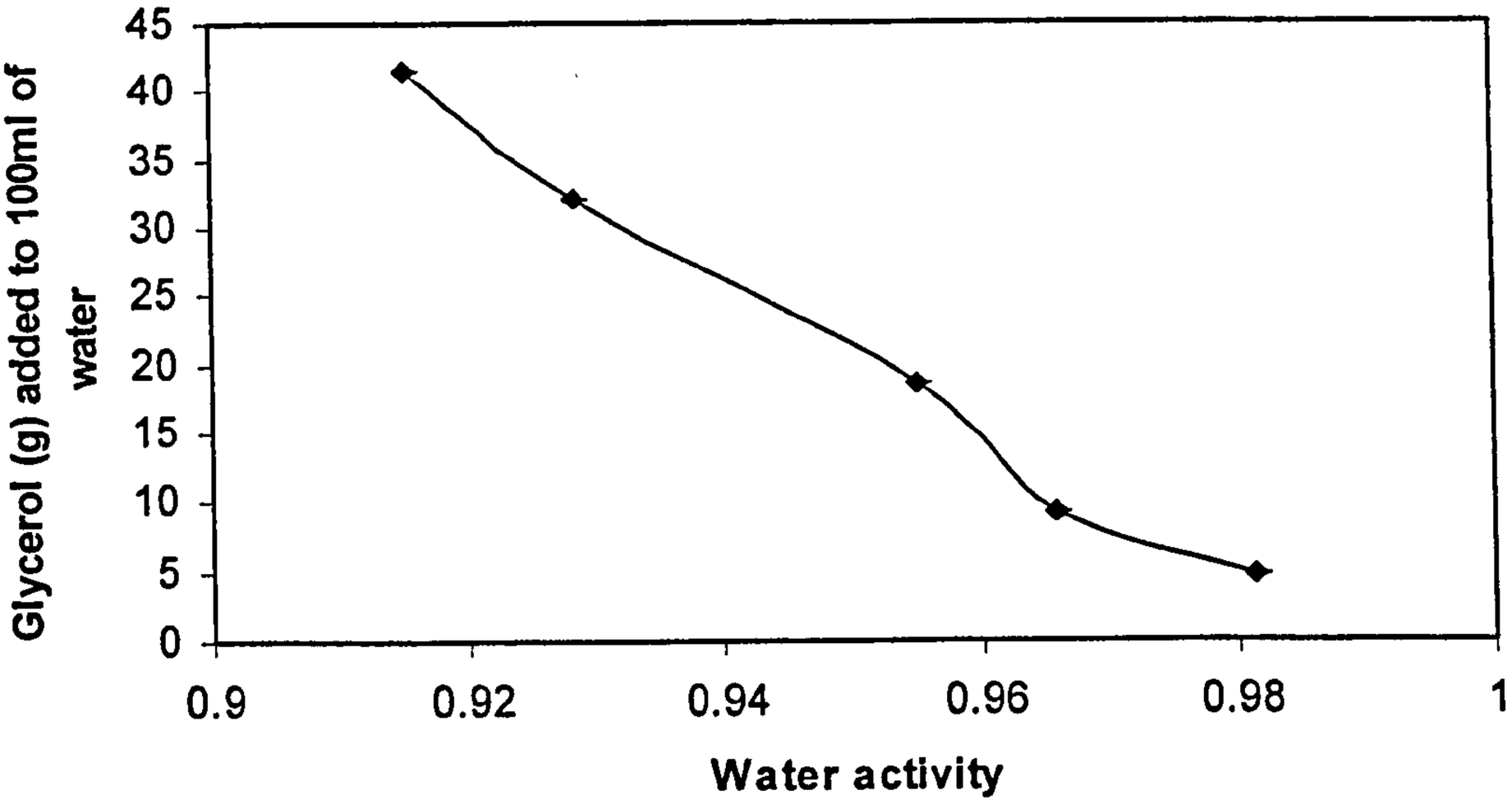


Figure 2.6. Water activity of 10 g fresh millet after they had been soaked in 5 ml of water/glycerol solutions with a range of glycerol concentration from 0 - 41.4 g/100 ml of water, autoclaved for 30 min and then left to equilibrate for 48 h (n=3). In all cases, the standard error of the means was smaller than the data points.

2.3.3 Temporal spore production by *M. anisopliae* on modified bulgar wheat

Figure 2.7 shows the number of spores produced by *M. anisopliae* over a 15 day fermentation period on bulgar wheat modified with water alone or water/glycerol solutions to different a_w levels (Appendix I). The ANOVA analysis of the data showed that all the 3 factors (time, a_w, ± glycerol) had a significant positive or negative effect ($P<0.001$) on spore production. For all treatments, spore production increased with time. For all times, more spores were produced on saturated (0.99 a_w) medium than on media of lower a_w, regardless of whether or not glycerol was used to modify the medium, and overall the lower the a_w the fewer spores were produced. However, after 15 days incubation, the quantity of spores produced on media modified to 0.96 a_w with water/glycerol solutions were similar to that produced on media modified to 0.98 a_w with either water/glycerol solution or water alone. Glycerol had either a positive or negative effect on spore production when it was used to modify the a_w, with an overall positive effect. The ANOVA analysis also showed (Appendix I) that the two way interactions between the main effects of added glycerol and a_w and between added glycerol and time were very significant ($P<0.001$) and between a_w and time was

significant ($P<0.05$). This means that none of the main factors acted independently, but the effect of each factor depended on the other two. However, the three way interaction between time, a_w and added glycerol was not significant.

2.3.4 Temporal spore production by *M. anisopliae* on modified millet

The temporal spore production by *M. anisopliae* on modified millet is shown in Figure 2.8. As with bulgar wheat, the ANOVA analysis of the data showed that all 3 factors (time, a_w, ± glycerol) had a significant positive or negative effect ($P<0.001$) on spore production (Appendix I). Again, for all treatments, spore production increased with time. For both times the number of spores produced on saturated (0.99 a_w) medium was higher than on the media of lowered a_w. In this experiment glycerol always had a positive statistically significant ($P<0.001$) effect on spore production which was more evident when compared with bulgar wheat. Significant interactions were only found between a_w and added glycerol ($P<0.001$) and between a_w and time ($P<0.05$). In this case the effect of glycerol was not dependent on time and vice versa.

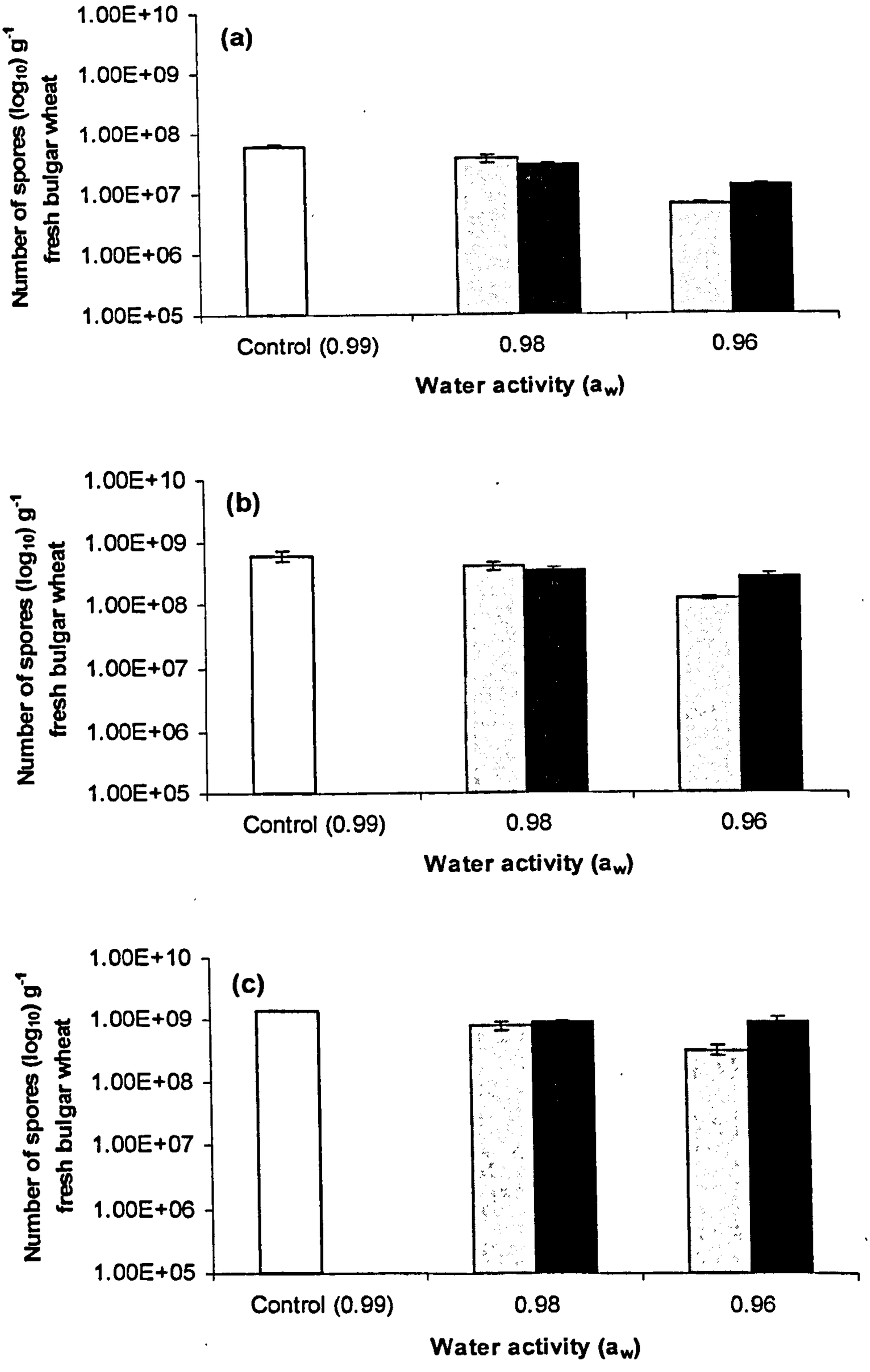


Figure 2.7. Conidial production on modified bulgar wheat at different times; (a) 5 d, (b) 10 d and (c) 15 d; in relation to a_w. The a_w was adjusted with either water alone (□) or with water/glycerol solution (■). The control treatment (□) was modified to saturation. Bars represent standard error of the means, (n=3).

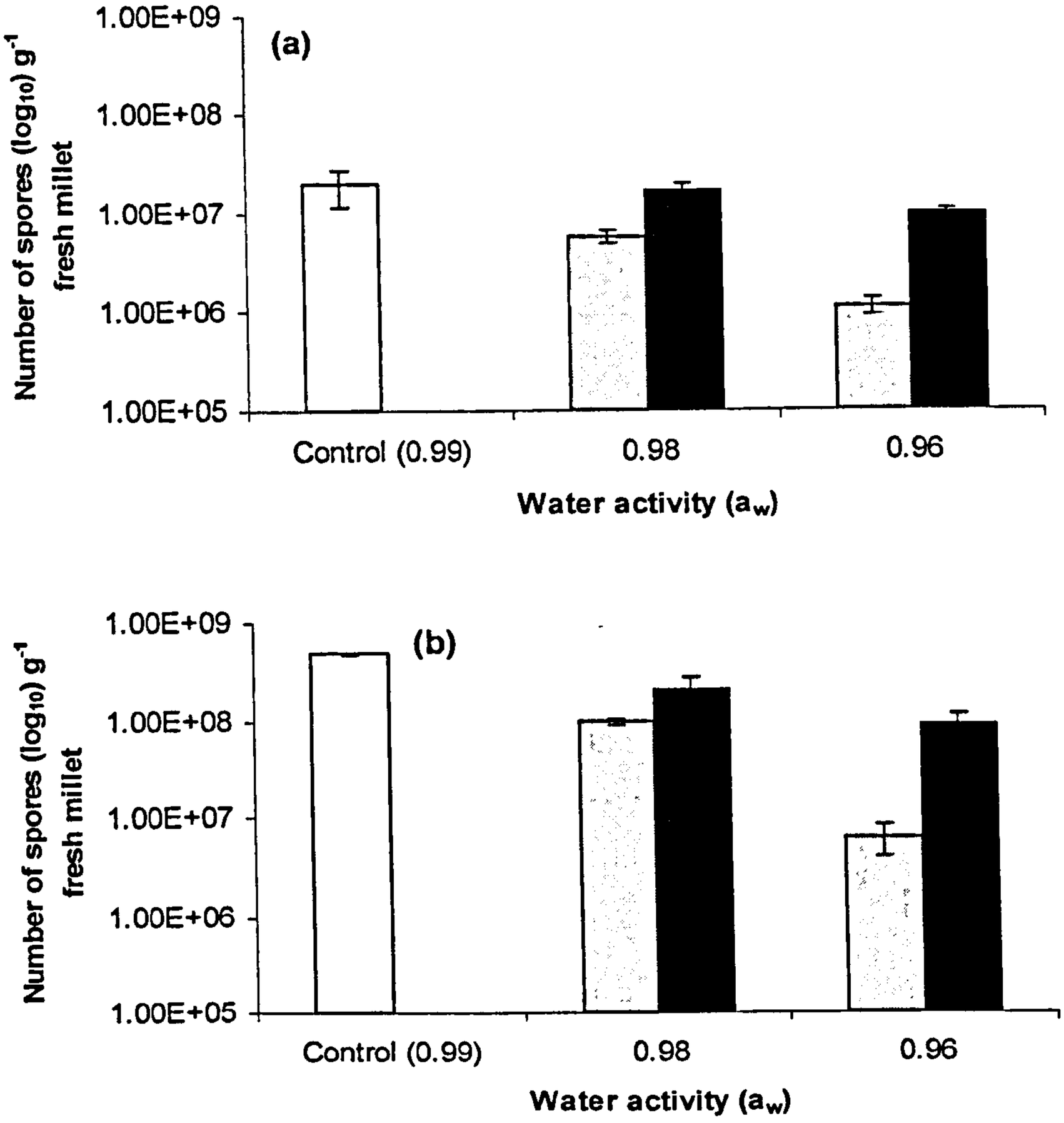


Figure 2.8. Conidial production on modified millet at different times; (a) 5 d and (b) 10 d; in relation to a_w. The a_w was adjusted with either water alone (□) or with water/glycerol solution (■). The control treatment (□) was modified to saturation. Bars represent standard error of the means, (n=3).

2.3.5 Polyol and sugar content of *M. anisopliae* conidia produced on modified bulgar wheat

Figure 2.9 shows the polyol content of conidia harvested after 10 and 15 days incubation on bulgar wheat. Quantities of spores produced after 5 days incubation were not enough to enable polyol analysis. Starting from the lowest to highest molecular weight polyols, the intracellular accumulation pattern was as follows. For both harvest times and for all treatments, the polyol glycerol was present in very low amounts (< 2.5 mg g^{-1} fresh weight). The only marked increase in endogenous erythritol content was observed in conidia produced at 0.96 a_w level modified with water alone and at 10 days

of incubation. Arabitol content of conidia from both culture ages (10 and 15 days) increased significantly ($P<0.05$) from 0.99 a_w (saturation) to 0.98 a_w when modification was with water alone. In low a_w treatments (0.96), arabitol content was significantly different compared to the saturated treatment only after 15 days of incubation and when water alone was used for modification.

Mannitol content of conidia from the 10-day-old cultures decreased progressively as the a_w decreased from saturation to 0.96 a_w with the decrease being less apparent in conidia from the 0.98 a_w treatment modified with a water/glycerol solution. However, after 15 days of incubation, mannitol content declined considerably compared to saturated conditions only in conidia from the lowest a_w (0.96) modified with water alone. It is interesting to note that the ratio of high (mannitol) to lower molecular weight polyols (glycerol, erythritol, arabitol) decreased with decreasing a_w for both 10 and 15-day-old cultures modified with water alone (Figure 2.10) with lower molecular weight polyols constituting more than 65% of the total polyol content at 0.98 and 0.96 a_w. For cultures modified with water/glycerol solution the decrease of the ratio of high (mannitol) to lower molecular weight (glycerol, erythritol, arabitol) polyols occured only for 10 day-old cultures with lower molecular weight polyols constituting more than 65% of the total polyol content only in cultures modified to 0.96 a_w (Figure 2.11).

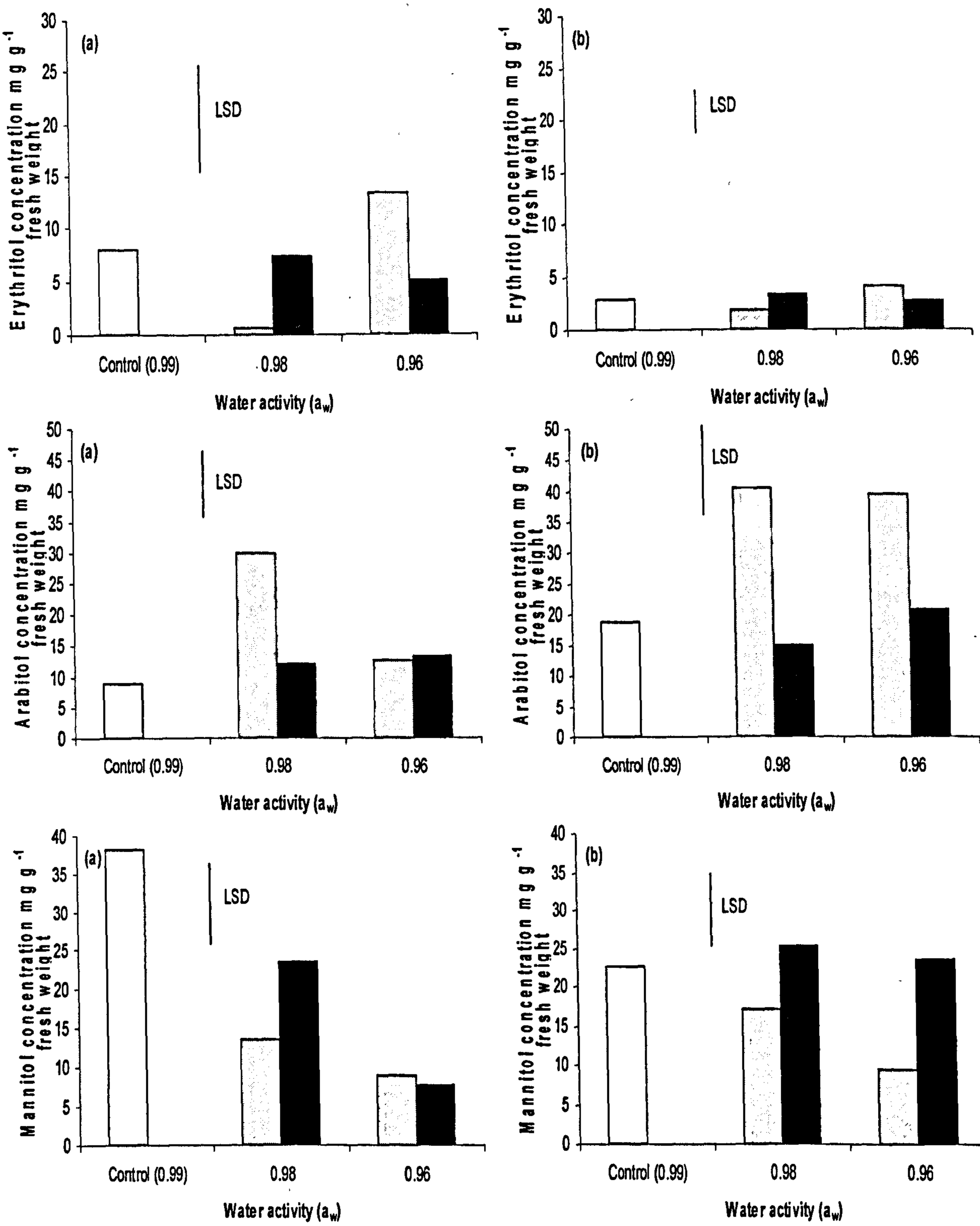


Figure 2.9. Polyol content of conidia harvested at (a) 10 and (b) 15 days after incubation on modified bulgar wheat, in relation to a_w. The a_w was adjusted with either water alone (□) or with water/glycerol solution (■). The control treatment (□) was modified to saturation. Bars indicate least significant differences (LSD) ($P<0.05$) of the means.

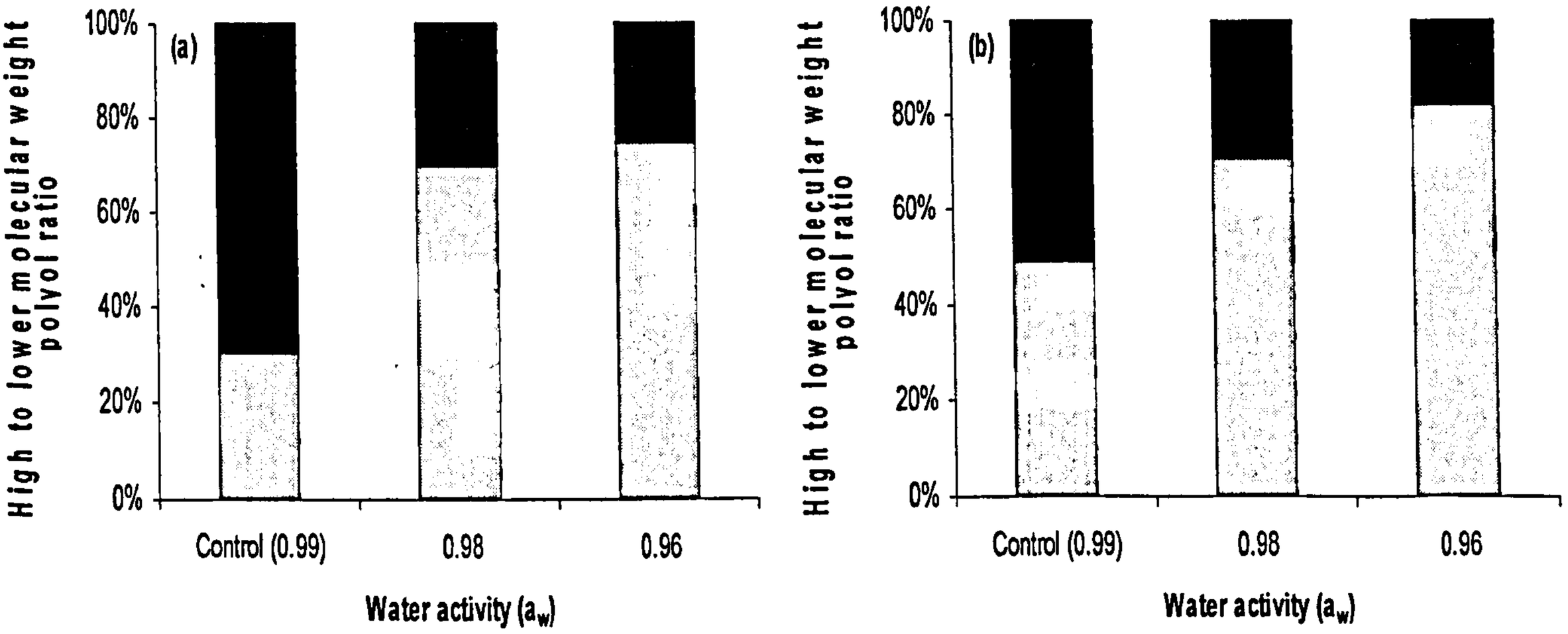


Figure 2.10. Ratio of high (mannitol;■) to lower (glycerol, erythritol, arabitol;■) molecular weight polyols in conidia harvested at (a) 10 and (b) 15 days after incubation on bulgar wheat modified with water alone, in relation to a_w.

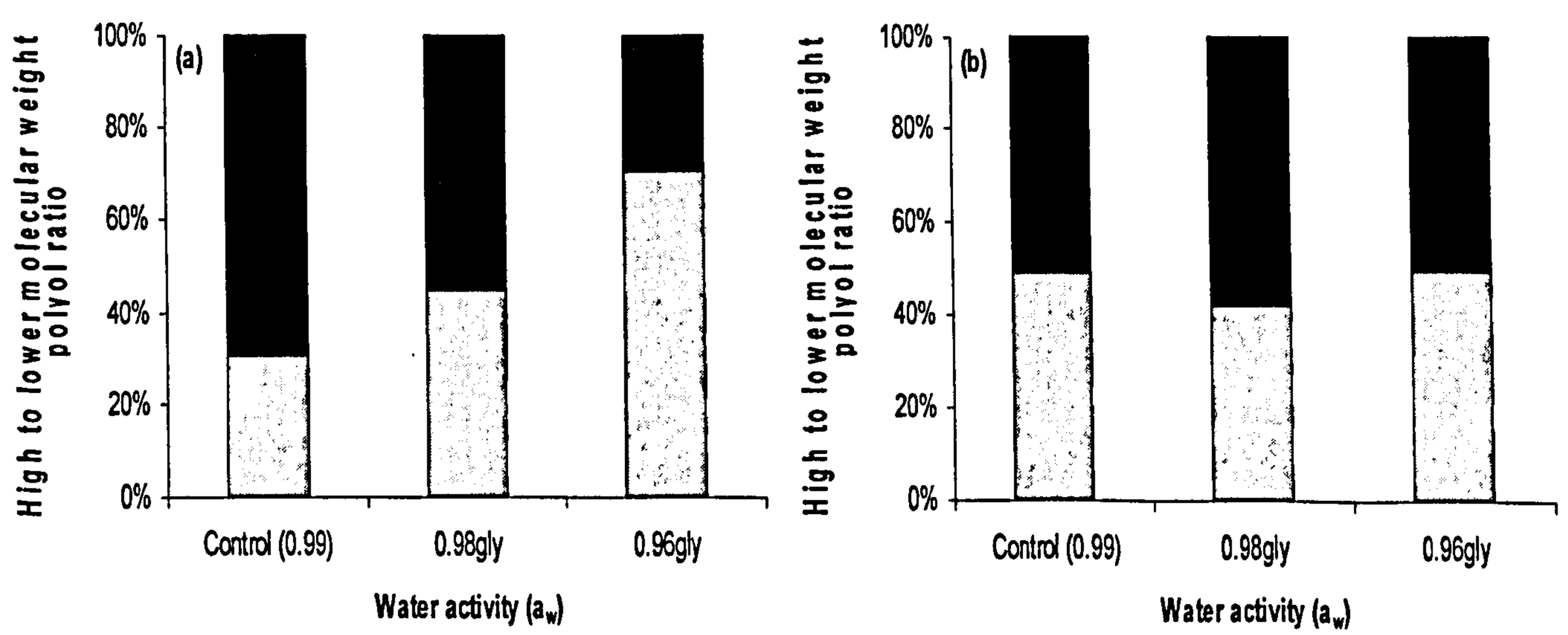


Figure 2.11. Ratio of high (mannitol;■) to lower (glycerol, erythritol, arabitol;■) molecular weight polyols in conidia harvested at (a) 10 and (b) 15 days after incubation on bulgar wheat modified with water/glycerol solution, in relation to a_w.

For both harvest times the sugar trehalose was present in very low amounts (< 2.5mg g^{-1} fresh weight) in spores from all treatments. Glucose content, generally, was not markedly different between treatments for both harvest times (Figure 2.12). However, glucose content of spores from the saturated treatment, decreased considerably with culture age and it was lower compared to all other 15-day-old treatments (Figure 2.12).

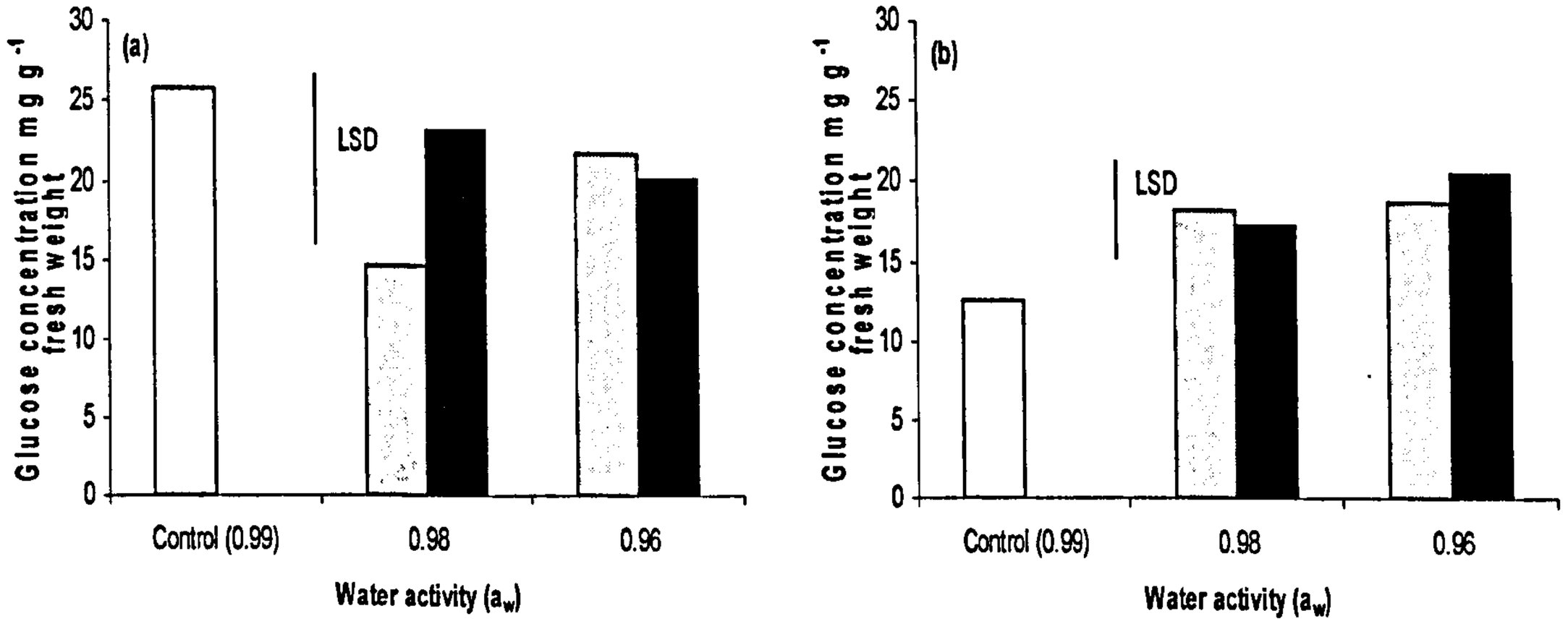


Figure 2.12. Glucose content of conidia harvested at (a) 10 and (b) 15 days after incubation on modified bulgar wheat, in relation to a_w. The a_w was adjusted with either water alone (□) or with water/glycerol solution (■). The control treatment (□) was modified to saturation. Bars indicate least significant differences (LSD) ($P<0.05$) of the means.

2.3.6 Sugar and polyol content of *M. anisopliae* conidia produced on modified millet

Enough quantities to allow polyol analysis were obtained at 10 days incubation and the results are shown in Figure 2.13. Glycerol was not detected in any of the treatments. Erythritol concentration was not markedly accumulated in any of the treatments and was lower than 5 mg g^{-1} fresh weight. Higher amounts of arabitol were observed in the treatments modified to 0.98 a_w with either water alone or water/glycerol solution, but no significant differences ($P<0.05$) were observed between any of the treatments. Mannitol content considerably decreased from saturation to 0.98 and 0.96 a_w level when the substrate was modified with water alone. As in the case with bulgar wheat substrate, the ratio of high (mannitol) to lower molecular weight (erythritol, arabitol) polyols decreased with decreasing a_w whether water alone (Figure 2.14) or water/glycerol

solution was used for modification (Figure 2.15), but this time lower molecular weight polyols constituted less than 50% of the total polyol content.

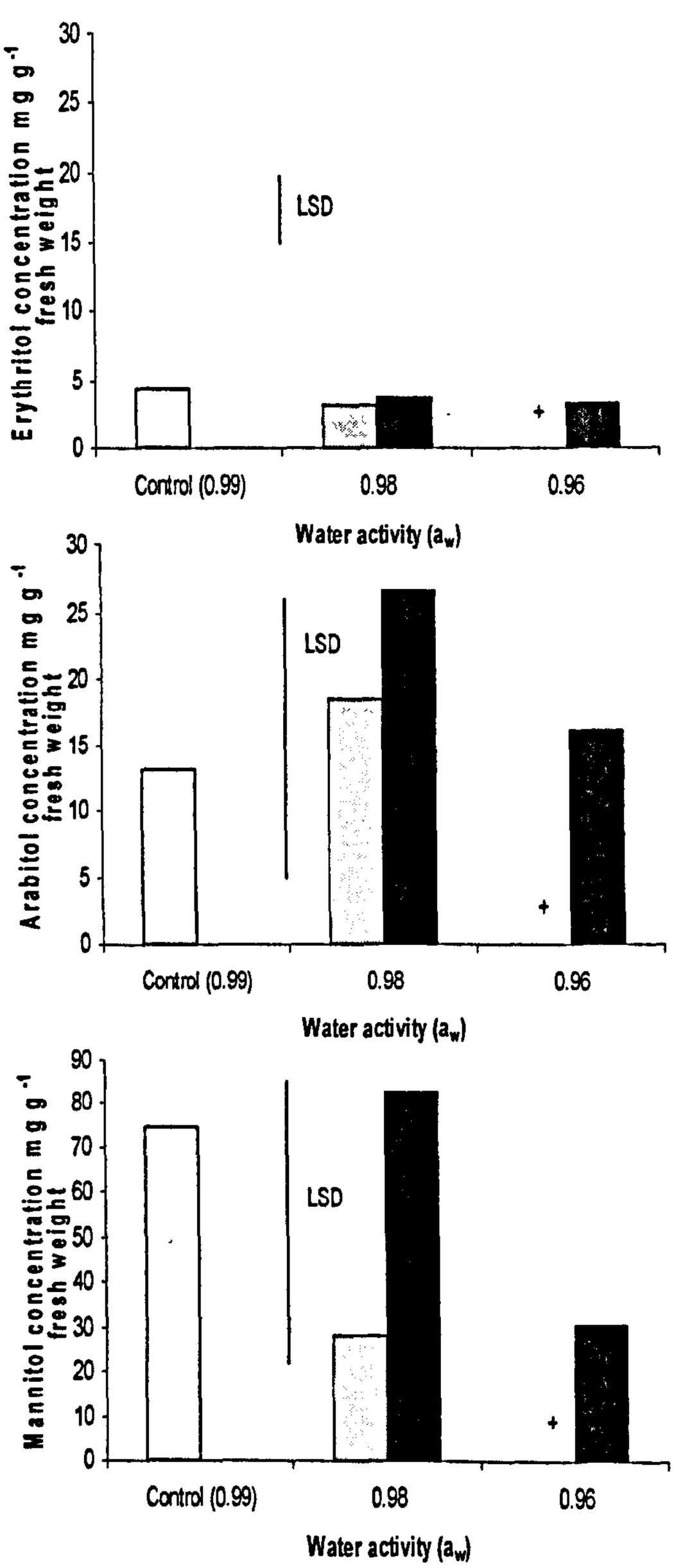


Figure 2.13. Polyol content of conidia harvested at 10 days after incubation on modified millet, in relation to a_w. The a_w was adjusted with either water alone (□) or with water/glycerol solution (■). The control treatment (□) was modified to saturation. Bars indicate least significant differences (LSD) ($P<0.05$) of the means. + Not enough spores for polyol analysis.

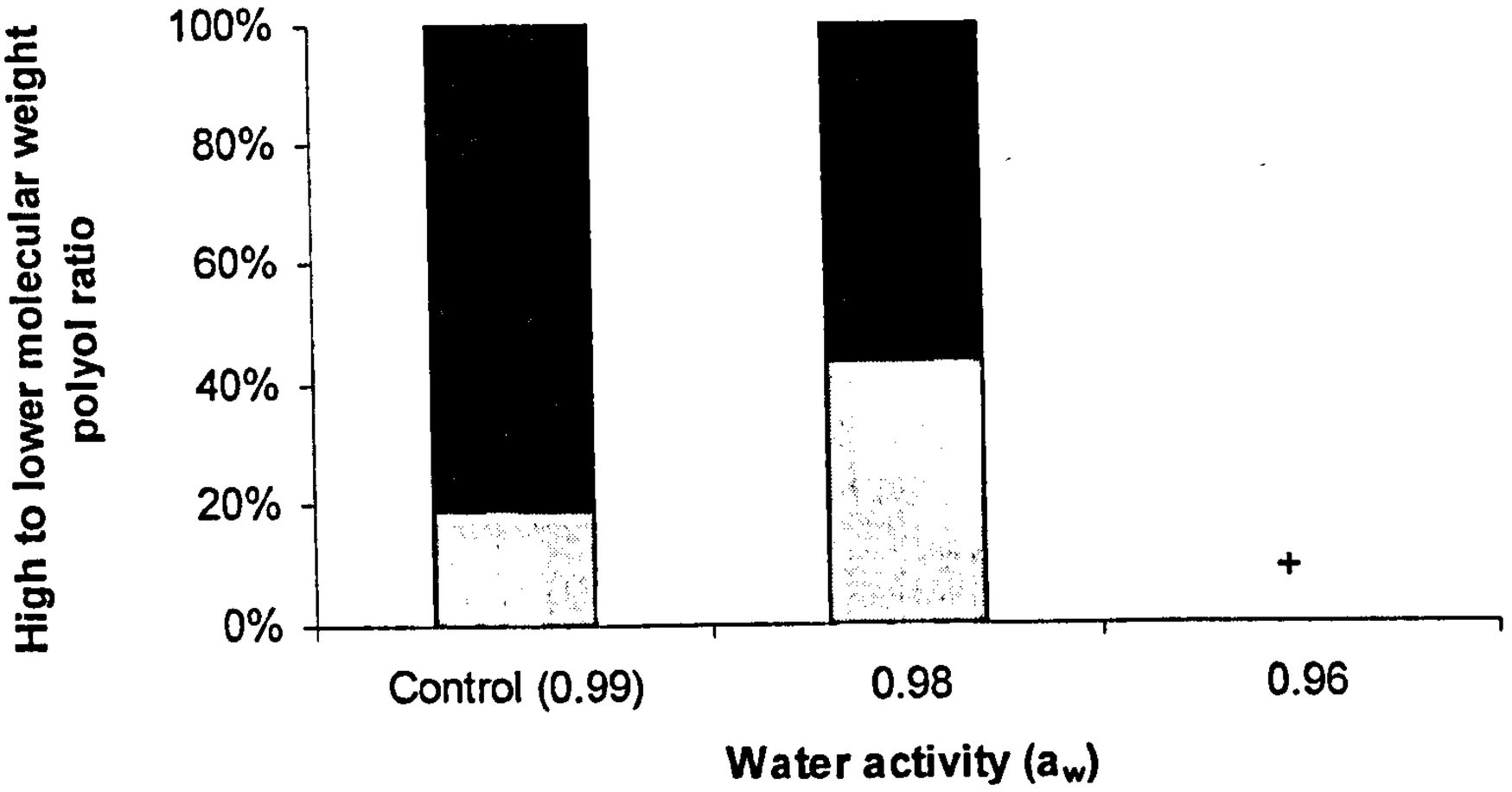


Figure 2.14. Ratio of high (mannitol;■) to lower (erythritol, arabitol;■) molecular weight polyols in conidia harvested at 10 days after incubation on millet modified with water alone, in relation to a_w. + Not enough spores for polyol analysis.

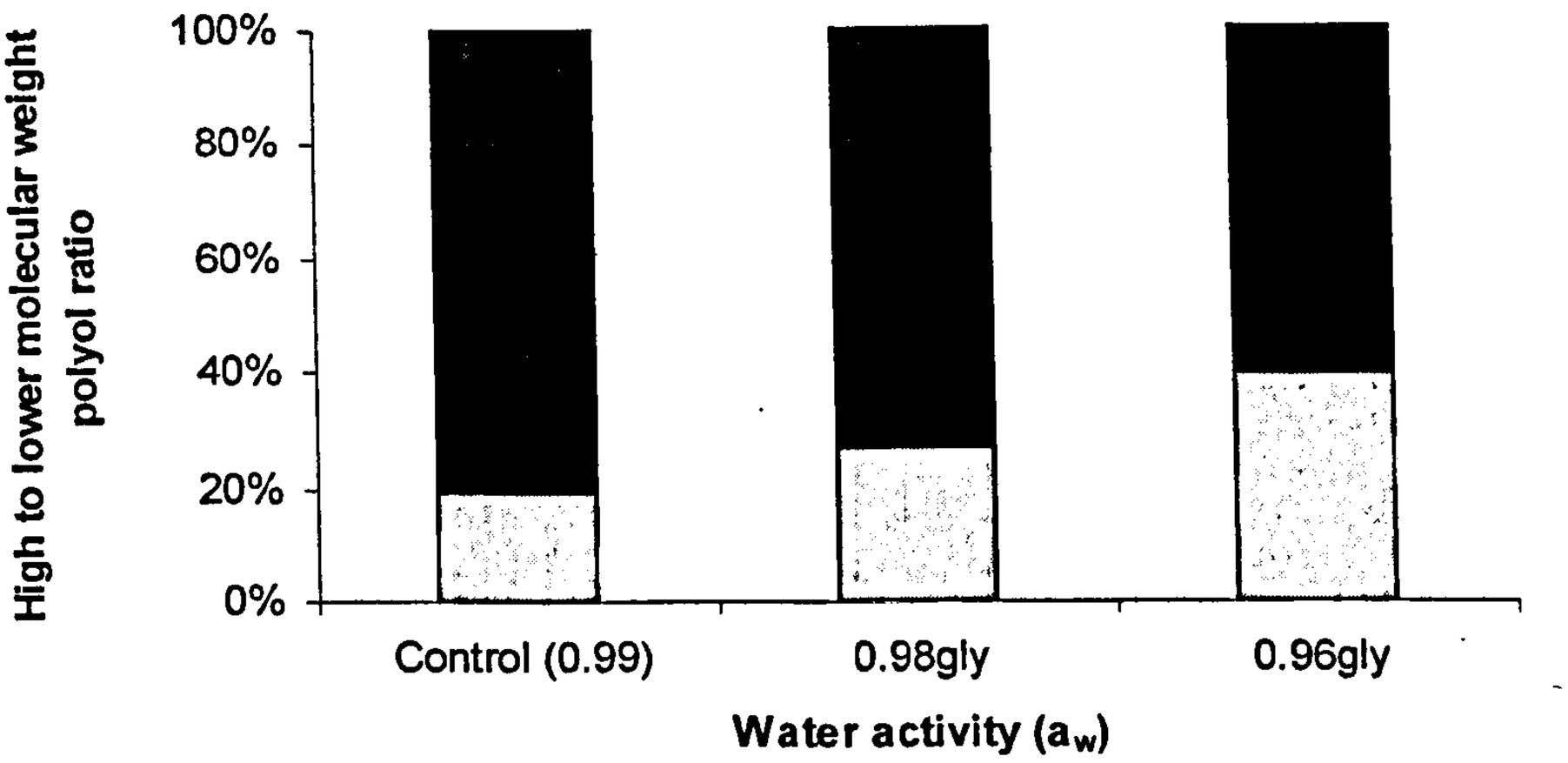


Figure 2.15. Ratio of high (mannitol;■) to lower (erythritol, arabitol;■) molecular weight polyols in conidia harvested at 10 days after incubation on millet modified with water/glycerol solution, in relation to a_w.

Trehalose was not detected in any of the treatments. Endogenous glucose concentration in conidia from the control treatment (saturated) at 10 days of incubation was the highest observed (31 mg g^{-1} fresh weight) in any of the treatments for both substrates (bulgar wheat, millet) and it significantly ($P<0.05$) decreased with decreasing a_w (Figure 2.16).

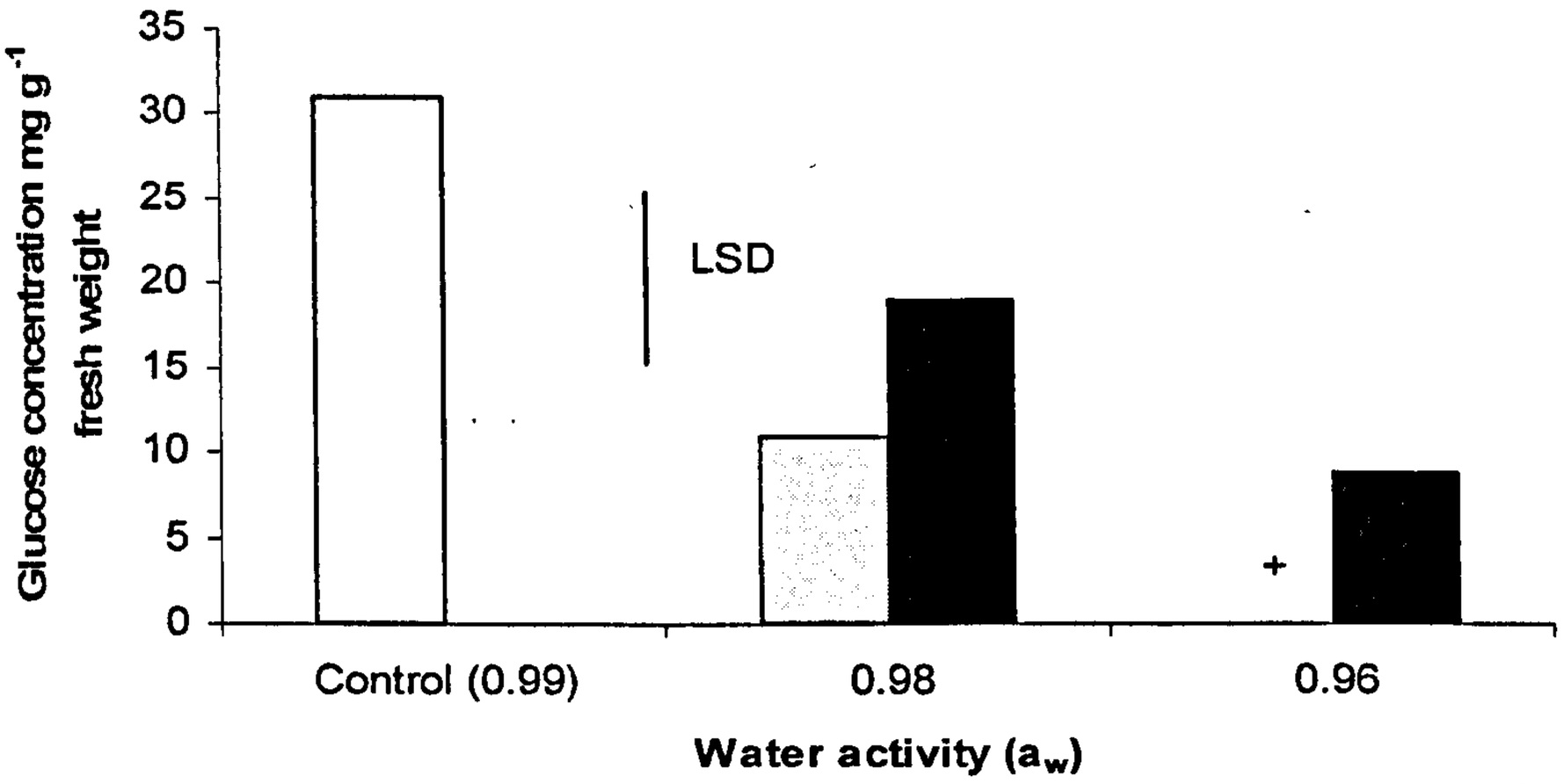


Figure 2.16. Glucose content of conidia harvested at 10 days after incubation on millet, in relation to a_w. The a_w was adjusted with either water alone (□) or with water/glycerol solution (■). The control treatment (□) was modified to saturation. Bars indicate least significant differences (LSD) ($P<0.05$) of the means.

2.3.7 Germinability of *M. anisopliae* conidia produced on modified bulgar wheat

Table 2.2 shows the percentage germination of conidia harvested from modified bulgar wheat after they had been spread plated and incubated on unmodified (0.998 a_w) and water-stressed (0.963 a_w) germination media. When germination was tested on unmodified germination medium, spores harvested after 5 days incubation from all treatments gave high percentage germination (> 92%). For all treatments, germination decreased with culture age on both unmodified and water-stressed germination media. For all harvest times, highest germination occurred with spores produced on bulgar wheat modified to the lowest a_w tested (0.96) with water alone, followed by spores produced on bulgar wheat modified with water/glycerol solution to the same a_w level. The reduction of germination with harvest time was more evident on water-stressed media compared to unmodified media and < 5% of spores harvested from all 15-day-old cultures germinated under these conditions.

Table 2.2. Germination of *M. anisopliae* conidia harvested 5, 10 and 15 days after incubation on modified bulgar wheat on two media, 0.998 and 0.963 a_w.

Conidia were incubated for 14 h on unmodified germination media (0.998 a_w) and for 36 h on water-stressed germination media (0.963 a_w) before measurements of germination were made. Values represent mean percentage germination of conidia ± standard error from 3 replicates (100 conidia per replicate). Least significant differences (LSD) ($P<0.05$) between values from different treatments are shown for each column.

Treatment	**PERCENTAGE GERMINATION**					
	a_w	**0.998**			**0.963**	
	Harvest time(d)					
	5	**10**	**15**	**5**	**10**	**15**
0.988	92±3.61	84±2.31	70±4.51	46.6±3.84	27.3±7.86	<5
0.980	96.6±0.88	93.6±0.88	69±4.58	47.3±8.82	26±1.53	<5
0.980 gly	98.3±0.33	95.3±0.66	71±7.42	43.6±4.48	28.6±4.09	<5
0.960	98.6±0.33	94±2.31	91.6±1.66	75±5.29	37±1.15	<5
0.960 gly	98.6±0.33	95.6±0.88	85±5.51	56.6±2.18	26.6±0.33	<5
LSD	5.29	5.31	16.36	16.96	12.67	

The germ tube length of conidia harvested from modified bulgar wheat after they had been spread plated and incubated on unmodified (0.998 a_w) and water-stressed (0.963 a_w) germination media is shown in Table 2.3. Overall, conidia from lower a_w produced longer germ tubes on either unmodified or water-stressed germination media compared to conidia from higher a_w with the only exception being the spores from the 15-day-old cultures modified to 0.96 a_w with water/glycerol solution. For all harvest times and for both germination media, conidia from bulgar wheat modified to 0.96 a_w with water alone produced significantly ($P<0.05$) longer germ tubes when compared to all treatments and in all cases except for spores from 10-day-old bulgar wheat cultures modified to 0.98 a_w with water/glycerol solution germinating on water-stressed media. As for germination, for all treatments germ tube lengths were reduced with harvest time. It is interesting to note that for all harvest times and on unmodified germination medium, spores from treatments modified with water/glycerol solutions produced

considerably shorter germ tubes than the treatments of the equivalent a_w but modified with water alone. The same trend but less obvious was observed on water-stressed germination medium.

Table 2.3. Germ tube length (μm) of *M. anisopliae* conidia harvested 5, 10 and 15 days after incubation on modified bulgar wheat on two media, 0.998 and 0.963 a_w.

Conidia were incubated for 14 h on unmodified germination media (0.998 a_w) and for 36 h on water-stressed germination media (0.963 a_w) before germ tube extension was measured. Values represent mean germ tube length (μm) of conidia ± standard error from 3 replicates (3x25 germinated conidia per replicate). Least significant differences (LSD) ($P<0.05$) between values from different treatments are shown for each column.

Treatment	**GERM TUBE LENGTH (μm)**					
a_w	**0.998**			**0.963**		
	Harvest time(d)					
	5	**10**	**15**	**5**	**10**	**15**
0.988	95.20±1.14	72.85±1.65	55.33±2.26	25.43±1.55	14.38±0.83	12.07±0.48
0.980	149.23±2.42	100.48±3.63	75.6±2.16	29.89±4.32	20.07±1.43	11.67±0.22
0.980 gly	134.06±2.17	89.20±2.76	65.87±2.66	27.23±0.52	22.98±1.83	11.67±0.30
0.960	200.73±4.45	132.47±4.98	85.93±3.06	42.50±2.18	25.54±2.27	16.42±1.67
0.960 gly	160.64±4.33	114.59±5.13	58.27±2.59	31.51±1.60	19.22±0.63	11.50±0.66
LSD	10.00	12.18	8.08	7.55	4.83	2.67

The results of the correlation between the number of spores produced on a modified bulgar wheat treatment and germinability of the spores produced are shown in Table 2.4. There was a strong negative correlation between the number of spores produced on a modified bulgar wheat treatment and the germinability (expressed either as percentage germination or germ tube length) of the corresponding spores produced (Pearson coefficient – 0.75). Pearson's correlation coefficient (r) for continuous (interval level) data ranges from -1 to +1. Positive correlation indicates that both variables increase or decrease together, whereas negative correlation indicates that as one variable increases, so the other decreases, and vice versa.

Table 2.4. Correlation between the number of spores produced on a modified bulgar wheat treatment and viability of the spores produced.

Type of germination medium	Expression of viability	Pearson coefficient	P
Unmodified	Percentage germination	- 0.65	< 0.001
Unmodified	Germ tube length	- 0.70	< 0.001
Stressed	Percentage germination	- 0.75	< 0.001
Stressed	Germ tube length	- 0. 71	< 0.001

2.3.8 Germinability of *M. anisopliae* conidia produced on modified millet

Table 2.5 shows the percentage germination of conidia harvested from modified millet after they had been spread plated and incubated on unmodified (0.998 a_w) and water-stressed (0.963 a_w) germination media. For either early harvested (5 days incubation) or late harvested (10 days incubation) spores there were no significant differences ($P<0.05$) in the percentage germination between all treatments when examined on unmodified germination medium. On water-stressed medium, however, and for both harvested times, spores from the 0.96 a_w treatment modified with water alone gave the highest germination. Additionally, although germination of conidia from all treatments, as in the case of bulgar wheat, decreased with harvest time, late harvested conidia from the 0.96 a_w treatment modified with water alone retained quite high germinability (> 55%) which was significantly different ($P<0.05$) from all the other treatments. On water-stressed medium it was also evident that spores from the treatments modified with water/glycerol solutions gave lower percentage germination compared to the spores from treatments with equivalent a_w but modified with water alone.

Table 2.5. Germination of *M. anisopliae* conidia harvested 5, and 10 days after incubation on modified millet on two media, 0.998 and 0.963 a_w.

Conidia were incubated for 14 h on unmodified germination media (0.998 a_w) and for 36 h on water-stressed germination media (0.963 a_w) before measurements of germination were made. Values represent mean percentage germination of conidia ± standard error from 3 replicates (100 conidia per replicate). Least significant differences (LSD) ($P<0.05$) between values from different treatments are shown for each column.

Treatment	PERCENTAGE GERMINATION			
	a_w 0.998		0.963	
	Harvest time(d)			
	5	10	5	10
0.988	95.3±1.76	89±2.08	35.6±6.64	17.3±2.18
0.980	95.6±0.66	95±0.57	56±7.02	36.3±9.35
0.980 gly	95.6±0.88	87.3±4.63	39.3±1.76	22.6±4.63
0.960	97.3±1.20	85.6±4.48	61.3±7.51	55±3.46
0.960 gly	98.3±0.66	87±1.00	54.3±3.28	19.3±5.81
LSD	3.53	9.34	18.13	17.89

Table 2.6 shows the germ tube length of conidia harvested from modified millet after they had been spread plated and incubated on unmodified (0.998 a_w) and water-stressed (0.963 a_w) germination media. For both unmodified and water-stressed media, spores from the 0.96 a_w treatment modified with water alone produced remarkably longer germ tubes compared to all treatments except when compared to spores from the 5-day-old cultures modified to 0.96 a_w with water/glycerol solution. Although germ tube length of spores of all treatments was reduced with harvest time, the reduction was much less with spores from the 0.96 a_w treatment modified with water alone.

Table 2.6. Germ tube length of *M. anisopliae* conidia harvested 5, and 10 days after incubation on modified millet over two a_w levels.

Conidia were incubated for 14 h on unmodified germination media (0.998 a_w) and for 36 h on water-stressed germination media (0.963 a_w) before germ tube extension was measured. Values represent mean germ tube length (µm) of conidia ± standard error from 3 replicates (3x25 germinated conidia per replicate). Least significant differences (LSD) (P<0.05) between values from different treatments are shown for each column.

Treatment	GERM TUBE LENGTH (µm)			
	a_w	0.998	0.963	
	Harvest time(d)			
	5	10	5	10
0.988	33.33±0.87	22.97±0.84	16.40±0.67	12.37±0.19
0.980	44.6±1.36	26.37±3.09	21.7±0.25	15.27±0.81
0.980 gly	48.27±1.59	23.17±1.29	20.87±0.83	12.77±1.02
0.960	57.27±4.99	54.40±7.41	23.13±2.17	20.90±0.35
0.960 gly	50.87±3.21	23.90±2.35	23.77±2.14	14.27±0.73
LSD	8.95	11.99	3.48	2.18

The results of the correlation between the number of spores produced on a modified millet treatment and viability of the spores produced are shown in Table 2.7. The correlation, was again high and negative (pearson coefficient -0.66) except when viability was examined on unmodified germination media and expressed as percentage germination (pearson coefficient -0.35).

Table 2.7. Correlation between the number of spores produced on a modified millet treatment and viability of the spores produced.

Type of germination medium	Expression of viability	Pearson coefficient	P
Unmodified	Percentage germination	- 0.35	0.055
Unmodified	Germ tube length	- 0.61	< 0.001
Stressed	Percentage germination	- 0.60	< 0.001
Stressed	Germ tube length	- 0. 66	< 0.001

2.4 DISCUSSION

For the successful development of a BCA it is important that studies are focused on developing protocols which use readily available cheap raw materials and maximise not only propagule yield but also propagule fitness. Propagule fitness is translated into high biocontrol efficacy even under sub-optimal conditions, desiccation tolerance and stability as a formulated preparation. A considerable number of studies have shown that manipulation of the production medium by changing environmental and chemical conditions, can have a profound effect on the quantity and quality of spores produced (Hallsworth & Magan, 1995; Cliquet & Jackson, 1999; Pascual *et al.*, 1999; Frey & Magan 2001; Jackson *et al.*, 2003). It is therefore possible by exploiting such manipulations to develop a production optimisation strategy for biopesticide production.

On both types of solid medium used (bulgar wheat and millet), the effect of a_w on spore production was significant and decreasing the a_w the number of spores produced was also decreased. Reduced mycelial growth with decreasing a_w has been reported previously. For example, Inch & Trinci (1987) reported a linear relationship between the reduction in growth of *P. farinosus* and the decrease in a_w from 0.98 to 0.94. They attributed this relationship to the increased energy requirements for osmoregulation at lower water activities. Previous studies of growth rates of *M. anisopliae* on agar media with different carbohydrate concentration and composition, showed that growth was

optimum at a_w values between 0.975 and 0.990 with a progressive reduction as the water availability was reduced to < 0.97 a_w (Hallsworth & Magan, 1994b). Growth rate was found to depend not only on the a_w of the medium but also on the type of carbohydrate source used to adjust the a_w, suggesting that when more utilisable types of carbohydrates are provided, optimum growth can occur over a wider range of a_w. In the present study with solid substrates, the optimum spore production occurred when water was freely available (0.99 a_w). The decrease in spore production with decrease in a_w could mainly be due to two reasons. Firstly, the higher amount of water used to modify the substrates to saturation compared to lower a_w levels, caused more swelling of the substrate particles enabling easier penetration by the fungus and more effective utilisation of the substrate. Secondly, the reduction in growth rates with decreasing a_w resulted in delayed sporulation. Gonzalez *et al.* (1988) have shown that in solid substrate fermentation of *P. chrysogenum*, of all fermentation conditions, the initial water moisture level is among the most critical.

On the solid substrate media of the same a_w level, at least as many (bulgar wheat 0.98 a_w) or markedly more (millet 0.98 a_w and 0.96 a_w both substrates) spores were produced when the a_w was modified with glycerol/water solutions than with water alone. The same trend was observed with the spore production of the fungal biocontrol agent *Epicoccum nigrum* on two types of wheat grain (Pascual *et al.*, 1999). It is more likely that this was because the solid media modified with water/glycerol solutions were more swollen compared to the media modified with water alone (the volume of water/glycerol solutions was the same as the amount of water used for saturation of the media) and not due to glycerol utilisation as a carbon source. This difference was more marked in the case of millet where the hard shell of the grains could have considerably prevented the fungus from easy access when small volumes of water were used (0.98 and 0.96 a_w). Evidence to support the non-utilisation of glycerol as a carbon source by *M. anisopliae* comes from two comparable studies on the growth rates on agar media modified with KCl or glycerol to a range of a_w levels (Hallsworth & Magan, 1994a,b). When KCl was used, growth rates were at least as high as in the case with glycerol modification. Additionally, it was on glycerol-modified agar media that growth rates of *M. anisopliae* was mostly affected by water-stress than on agar media modified with other sources of

carbohydrates (starch, trehalose, glucose) (Hallsworth & Magan, 1994b), suggesting that glycerol was not predominantly utilised if at all, to provide the increased energy requirements at low a_w.

Higher spore production occurred on bulgar wheat grains compared to the corresponding treatments of millet grains. Bulgar wheat provides a more accessible substrate than millet, but needs to be investigated whether it also provides a more optimum nutritional environment leading to faster growth and higher conidiation.

In conditions of water-stress, compatible solutes are accumulated by microorganisms to reduce their internal water potential (Jennings, 1995). These compounds have been associated with accelerated germination, enhanced pathogenicity, and improved storage life of fungal propagules (Hallsworth & Magan, 1994c; Hallsworth & Magan, 1995; Pascual *et al.*, 2003). It has been suggested that the polyol content of fungal cells is composed of progressively lower molecular weight polyols as the a_w of the culture medium is reduced (Hallsworth & Magan, 1994a,b), but the nutritional composition of the medium can also affect the polyol endogenous composition (Adler *et al.*, 1982). At a given concentration, lower molecular weight polyols are more effective in reducing intracellular water potential than high molecular weight polyols (effect of second thermodynamic law). Therefore the following polyol sequence, starting from the one with the lowest molecular weight, in terms of effectiveness in osmoregulation should be expected: glycerol<erythritol<arabitol<mannitol. In this study mannitol decreased and arabitol increased with decreasing a_w. As a result of this shift the ratio of high molecular weight polyol mannitol to lower molecular weight polyols (erythritol and arabitol) decreased with decreasing a_w. This was most pronounced when the substrates (bulgar wheat and millet) were modified with water alone rather than with water/glycerol solution, and it was not observed after 15 days of incubation with the water/glycerol modified bulgar wheat treatments. Growth was faster (personal observations) and more spores were produced on substrates modified with water/glycerol solution, and this could have resulted in a higher production of metabolic water and subsequent increase of the substrate a_w, resulting in more even a_w levels between those treatments especially

at a later culture age. This could explain the less apparent or no shift from mannitol to arabitol accumulation in those treatments.

Glycerol endogenous content remained low (< 2.5 mg g^{-1} fresh weight) in all conidial treatments, regardless of substrate, culture age and modification with water alone or water/glycerol solution. Previous studies with the effect of carbohydrate type and concentration in agar-based media on polyol accumulation in *M. anisopliae* conidia showed that high glycerol amounts were detected only when glycerol was supplied exogenously (Hallsworth & Magan, 1994b). It is possible therefore that this polyol is not synthesised by this fungus and it can only be taken up when it is provided exogenously. Endogenous amounts of erythritol exceeded 10 mg g^{-1} fresh weight of spores only when bulgar wheat was modified to 0.96 a_w with water alone. This is consistent with previous studies with semi-solid *M. anisopliae* cultures modified with either ionic (KCl) or non-ionic (glycerol) solutes, where erythritol concentration reached the same amount in conidia produced at a a_w lower than 0.96 (Hallsworth & Magan, 1994a,b). The predominant polyols in this study was the high molecular weight mannitol and the lower molecular weight arabitol. The highest amount of arabitol (40 mg g^{-1} fresh weight) was recorded at 0.98 a_w modification of bulgar wheat with water alone and the highest amount of mannitol at 0.98 a_w modification of millet with water/glycerol solution. Studies with polyol accumulation in fungi and yeasts under water-stressed conditions, have shown that the pattern of polyol accumulation is dependent not only on the a_w itself but also on the type of a_w-modifying solute (Adler *et al.*, 1982; Hallsworth & Magan, 1994b; Hallsworth & Magan, 1995) and sometimes being more dependent on the solute used to adjust the a_w than on the a_w itself (Abadias *et al.*, 2001). Hallsworth & Magan (1994b), reported accumulation of arabitol in *M. anisopliae* conidia produced on agar cultures modified with glucose or trehalose but negligible amounts when glycerol was the a_w-modifying solute. They also reported that the accumulation of the disaccharide trehalose varied not only with the solute used to modify the a_w of the medium but also between microorganisms. In their study, trehalose was present at very low amounts (< 2.5 mg conidia g^{-1}) in *M. anisopliae* conidia when glucose, glycerol, or starch was the a_w-modifying solute and at higher concentrations (between 2.5-10 mg conidia g^{-1}) when trehalose was used for a_w-modification. In the

present study, trehalose was also present in very low amounts (< 2.5 mg conidia g^{-1}) in conidia from all treatments. The disaccharide trehalose can replace water in membranes at reduced a_w (Crowe *et al.*, 1984) and is known to stabilise enzyme structure during desiccation (Carpenter & Crowe, 1988). This disaccharide has been found to enhance desiccation tolerance of fungal spores providing in this way, potential for improving the storage life of fungal inocula (Harman *et al.*, 1991).

Germination efficiency of conidia harvested from both bulgar wheat and millet differed markedly depending on the treatment and the harvest time. For both solid substrates and for up to 10 day-old-cultures, increased water-stress resulted in the production of conidia with increased % germination and/or longer germ tubes both under conditions with fully available water and under water-stress conditions. When substrate modification was with water alone, the germination efficiency of produced conidia was better than when the substrates were modified with water/glycerol solution. Although this improved germination of conidia produced under increasing water-stress is linked with the observed shift in the endogenous polyol content from the high molecular weight mannitol to the low molecular weight arabitol, it is not possible to confirm that the two events are strictly interlinked. Two reasons exclude such a definite link. First, conidia from the 15-day-old bulgar wheat cultures, regardless of the substrate a_w, gave a lower than 5% germination on water-stress agar, although conidia from the 0.96 a_w treatment modified with water alone contained more than 65% low molecular weight polyols of the total polyol fraction. Secondly, the endogenous total polyol content of 10-day-old conidia produced on millet modified to 0.98 and 0.96 a_w was comprised of less than 50% of low molecular weight polyols, yet it gave a higher % germination under water-stress conditions compared to the respective bulgar wheat treatment in which low molecular weight polyols consisted more than 65% of total polyol content.

It was observed that when conidia were produced in low numbers germinated better than when they were produced in higher numbers. The number of spores produced on a treatment was strongly and negatively correlated to spore germinability. It seems likely that when the first spores are produced more nutrients are channelled into them and progressively the nutrient availability drops with a concomitant change in content of

subsequent spores. Nutritional content of spores can affect the speed with which they germinate. In many fungi, the initial events in spore germination require endogenous supplies of amino acids for protein synthesis while subsequent germ tube extension is dependent on exogenous sources of carbon and nitrogen (Van Etten *et al.*, 1976; Penington *et al.*, 1989). The same holds true with *M. anisopliae* conidia (Dillon & Charnley, 1990). It is therefore possible that when fewer spores are produced on a solid substrate, higher concentrations of nutrients are available to them which might contribute to higher concentrations of endogenous protein providing the amino acid pool necessary for protein synthesis required for subsequent germination upon availability of exogenous nutrient supply. Rapid germination of conidia of *M. anisopliae* soaked in distilled water when they were provided with an exogenous nutrient source was associated with increased protein synthesis compared to non-soaked conidia (Dillon & Charnley, 1990). Similar observations have been reported with the production of the fungal pathogen of weeds *Colletotrichum truncatum* in liquid culture of different C/N ratios (Jackson & Schisler, 1992). Conidia of this fungus produced in cultures with higher nitrogen availability (10:1 C/N ratio) contained more protein and germinated faster than conidia produced in cultures of lower nitrogen availability (30:1 or 80:1 C/N ratio). In an earlier study with the same fungus, it was also reported that a higher proportion of conidia produced in 10:1 medium contained two, rather than one, nuclei per conidium, a condition that could indicate the completion of the sequence of germination events preceding the emergence of a germ tube (Schisler *et al.*, 1991). Lane *et al.* (1991a,b) on the other hand, reported that *B. bassiana* blastospores produced in nitrogen-limited liquid cultures germinated at the same rate as the ones produced when nitrogen was provided in higher amounts but it was the nitrogen-limited blastospores that kept their ability to germinate on agar medium longer after storage and that were more virulent. This result may appear to be the opposite with the case of *C. truncatum* when it is first looked at, but if it is examined in relation to the number of spores produced under the two different conditions of nitrogen availability then it might be in agreement. The fact that they found that under nitrogen-limited conditions less *B. bassiana* blastospores were produced than when nitrogen was available in higher amounts, could imply that the less blastospores are provided with more nitrogen. Such a relationship however cannot be made easily, especially if the nitrogen content of the

produced blastospores is not known, and such differences in results may indicate that cultural conditions affect different organisms in a different way. From the above discussion, it appears more likely that in the present study, the enhanced germinability of spores produced under lower a_w is mainly due to effect of a_w on reducing the number of spores allowing more nutrients to be channelled into the small number of spores. Analysis of the endogenous reserves of spores produced on different treatments would provide important information for supporting this hypothesis. Glucose content of conidia, however, has been analysed and it does not appear to account for increased germinability since no marked difference was found between treatments.

The type of solid substrate influenced not only the amount of produced spores, but also their germinability. For all treatments and for all harvest times, more spores were produced on bulgar wheat than on millet grains, yet the germinability (percentage germination and/or germ tube length) of conidia from bulgar wheat treatments were markedly higher than conidia from millet treatments (except for percentage germination on agar with water-stress for the cultures modified to 0.98 and 0.96 a_w with water alone). In relation to what has been discussed so far, that could suggest that the nutritional content of bulgar wheat is more favourable for conidial production of higher quality compared to millet, or that the physical properties of bulgar wheat grains allowed the fungus to access more nutrients. Care must be taken in interpreting comparative results between different types of solid substrates because utilisation of solid substrates by microorganisms is affected by many factors such as particle size, shape, surface-to-volume ratio, crystallinity, and porosity of the substrate, all factors that can influence the accessible surface area to both organism and enzymes (Murthy *et al.*, 1993). In liquid fermentations on the other hand, all the substrate is equally accessible to the organism.

For both solid substrates and for all treatments, conidial germinability decreased with increased culture age. That could be associated again with the increase in number of spores with time. Hall *et al.* (1994) studied the influence of culture age of four deutoromycetous entomogenous fungi on conidial germination and found that *M. anisopliae* conidia harvested from young (2-3 d old) Petri plate cultures germinated

more rapidly than those taken from older cultures. They suggested that first-formed conidia might differ from those formed subsequently from the same phialide and the physiological manifestation of this could be faster germination of such first-formed conidia. Although they found a ten-fold increase in spore production between days 3 and 5 incubation, they did not consider any correlation between number of produced spores and germinability. It could have been possible, as in the case of the present study with solid substrate fermentation, that less nutrients are available for the late produced spores compared to the first ones, in this way reducing their germinability with time. However, the fact that they did not observe any decrease in germinability with time for *B. bassiana* conidia leaves ground for physiological differences, at least partially, between early and late harvested spores to account for the reduction in germinability.

It should be pointed out here that although fast *in vitro* germination of fungal spores has often proved a good indication of their effectiveness as a BCA (Schisler *et al.*, 1991; Hallsworth & Magan, 1994c; Chandler *et al.*, 1994) studies must always examine whether good *in vitro* germinability translates into good *in vivo* pathogenicity and consider long term viability and persistence in storage. Fungi with a certain *in vitro* germinability may have increased or reduced *in vivo* germinability due to several factors interfering during the pathogenicity process, such as environmental factors (solar radiation, temperature, water availability and rainfall), host factors, and pathogen physiology (e.g. production of enzymes and toxins). For example, a higher percentage of *C. truncatum* spores produced in 30:1 C/N ratio medium, germinated on the leaves of the weed *Sesbania exaltata* than on cellophane membranes. It was suggested that leaf surfaces can provide nutrients that overcome spore germination inhibitors.

Chapter 3 LIQUID FERMENTATION

3.1 INTRODUCTION

Liquid fermentation as opposed to solid substrate fermentation has the advantages of being a controlled process, ease of product harvesting and short fermentation times (Deshpande, 1999). Although mycopathogenic fungi have commonly been reported to produce conidia in liquid cultures (Harman *et al.*, 1991; Pascual *et al.*, 1997; Frey & Magan, 2001), the usual production of entomopathogenic fungi when grown in liquid culture is mycelial fragments and blastospores, the latter being produced by budding off from the hypha. There are reports, however, of the formation of true conidia in liquid culture by the entomopathogenic fungi *B. bassiana* and *M. flavoviride* (Thomas *et al.*, 1987; Jenkins & Prior, 1993).

The main limitation with liquid fermentation is that submerged spores are usually short-lived and do not survive adverse environmental conditions as well as aerially produced conidia (Lane *et al.*, 1991a; Muñoz *et al.*, 1995; Pascual *et al.*, 2000b; Frey & Magan, 2001). Submerged blastospores produced by the entomopathogenic fungus *M. flavoviride* are totally devoid of the extra warty outer layer observed in the case of aerial conidia. Submerged conidia of this fungus possess this layer but to a lesser extent compared to aerial conidia (Jenkins & Prior, 1993). The same observations have been reported for the biocontrol fungus *T. harzianum* (Muñoz *et al.*, 1995). Additionally, aerially produced spores of *T. harzianum* were highly hydrophobic compared to submerged ones (Muñoz *et al.*, 1995). These properties make aerial conidia less vulnerable to environmental conditions. However, there is the potential to improve the stability of submerged produced spores. Methods reported include growing the fungus at reduced a_w (Jin *et al.*, 1991), by culturing the fungus under different carbon and nitrogen ratios (Lane *et al.*, 1991a), by manipulating the nutritional status of the liquid culture, especially as far as nitrogen source is concerned (Jackson *et al.*, 1997; Jackson *et al.*, 2003) or by storage in appropriate liquids such as 10% hydroxyethyl starch (Kleespies & Zimmermann, 1994).

A number of nutritional studies have been undertaken for improving the sporulation of *M. anisopliae* in liquid culture (Barnes *et al.*, 1975; Cambell *et al.*, 1978;1983; Kleespies & Zimmermann, 1998; Fargues *et al.*, 2001; Jackson *et al.*, 2003; Vega *et al.*, 2003). However, there is limited knowledge about the effect of cultural environmental parameters and the interaction with nutritional changes on the physiological changes of blastospores that could result in the production of spores with better quality. Taking this into consideration, and starting with a liquid medium (Adámek, 1963) that has been found the most favourable for *M. anisopliae* blastospore production (Fargues *et al.*, 2001), a number of optimization studies were carried out in order to improve blastospore production, quality and germination efficiency of *M. anisopliae*. To this end the following aspects were examined:

1. Optimum conidial inoculum size for blastospore production by evaluating a range of inoculum sizes.
2. The impact of water-stress imposed by different solutes on blastospore production and quality in terms of endogenous reserves and efficiency of germination under high and low water availability conditions. Determination of the optimum conditions (a_w, a_w-modifying solute and incubation time) where both high blastospore production and good germinability was identified.
3. The impact of hyposmotic shock and osmoprotection of modified blastospores on the retention of endogenous reserves and efficiency of germination.
4. Nitrogen screening for improved *M. anisopliae* blastospore production at the optimum conditions (a_w, a_w-modifying solute and incubation time) where both high blastospore production and good germination efficiency occurred.
5. The effect of pH under optimised nitrogen source conditions on *M. anisopliae* blastospore production, endogenous reserves, and germination.
6. Selection of the best treatments in terms of blastospore production, quality and germination efficiency for storage stability studies.

3.2 MATERIALS AND METHODS

3.2.1 Fungal species

As described in Section 2.2.1

3.2.2 Impact of conidial inoculum size on blastospore production by *M. anisopliae*

Nutritional studies testing the effect of different liquid media on blastospore production by *Metarhizium* spp. have shown that Adámek's medium (1963) supports high blastospore production (Kleespies & Zimmermann, 1992; 1998; Fargues *et al.*, 2001). This medium was used as standard to determine optimum conidial inoculum size and it was prepared as follows: Distilled water + 3% cornsteep solid (Sigma, U.K) was autoclaved for 30 min, and filtered through 2 layers of Lens cleaning tissue 105 (Whatman, 105). In the filtrate, 4% glucose (Sigma, U.K.), 4% yeast extract (LAB M) and 0.1% Tween 80 (Sigma, U.K) were added. The pH was adjusted to 6.8 using 40% NaOH (Sigma, U.K). The a_w of this medium was found to be 0.989 ± 0.03 using an Aqualab Series 3 (Aqualab, Labcell Ltd., Basingstoke, Hants, U.K.).

Preliminary experiments with Erlenmeyer flasks (250 ml) containing 50 ml of standard medium and inoculated with 0.5 ml of a range of conidial inoculum sizes from $5x10^5$ to $5x10^7$ spores ml^{-1}, showed that at 25°C and 180 r.p.m. incubation on a rotary shaker, very few or no blastospore formation occurred, while mycelium formation was extensive with inoculum sizes higher than $1x10^6$. When baffled Erlenmeyer flasks (250 ml Nalgene) where used instead, blastospore production increased markedly and determination of optimum conidial inoculum size was performed using conidial suspensions in the range of $5x10^5$ to $5x10^7$ spores ml^{-1} prepared by flooding 10-14 day-old MEA *M. anispoliae* cultures. Each inoculum size was tested in triplicate.

3.2.3 Impact of water-stress on blastospore production, endogenous reserves and ecological fitness

Media preparation

Adámek's medium was used as the control (umodified) medium. The control medium was modified to either 0.98, 0.97 or 0.96 a_w, by adding either KCl (2.24%, 3.73%, 7.45%), NaCl (1.5%, 2.9%, 5.25%) or PEG 200 (7%, 15%, 30%) to distilled water prior to the addition of cornsteep solid, glucose and yeast extract. After that, final volumes were measured and 3% of cornsteep solid was added and the same procedure as the one described in 3.2.2 for the unmodified medium preparation was followed. The a_w of all media was measured before autoclaving. The pH for all treatments was adjusted to 6.8 before autoclaving using 40% NaOH.

Culture conditions and quantification of blastospores

Each treatment was carried out in triplicate 250 ml baffled flasks (Nalgene) containing 50 ml of liquid medium. Flasks containing the liquid medium were autoclaved at 120°C for 15 mins and each one inoculated with 0.5 ml conidial suspension of $5x10^6$ conidia ml^{-1}. Conidia were collected from 10 to 14-day-old cultures grown on MEA at 25°C. Liquid cultures were incubated on a rotary shaker at 180 r.p.m. for up to 90 h for the control, and treatments adjusted to 0.98 a_w, for up to 96 h for the treatments adjusted to 0.97 a_w and for up to 120 h for the treatments adjusted to 0.96 a_w. The experiments were finalised after the aforementioned incubation times as the fungus started to grow on the surface of the culture. Blastospore counts using a haemocytometer were done every 6-12 h by filtering 1 ml of subsample from each replicate flask through one layer of Lens tissue.

Harvest of blastospores

After blastospore quantification and determination of the incubation time when highest yield occurred, the experiment was repeated and blastospores from each treatment were harvested at these incubation times. For the treatments adjusted to 0.98 a_w and 0.97 a_w, blastospores were harvested at 72 and 84 h incubation, whereas for the treatments adjusted to 0.96 a_w blastospores were harvested after 108 and 120 h incubation. The sampling times were chosen to include a sampling with a ± 12-hour-interval from the

highest spore yield in order to study the effect of culture age for each treatment independently. The content from a replicate flask was filtered through two layers of sterile lens tissue to remove mycelial fragments. The filtrate was centrifuged at 2700 r.p.m. for 10 min. After centrifugation the supernatant was used to determine the pH and then discarded. The resultant spore pellet was washed with 10 ml of sterile distilled water and re-centrifuged. The water was then decanted to clean centrifuge tubes and the blastospores were used for germination studies and intracellular polyol determination as described below. The water used to wash the blastospores was also submitted to polyol analysis.

Extraction and detection of polyols

Twenty to 50 mg of blastospores (fresh weight) from each treatment were used for polyol extraction. The method for polyol extraction and quantification has been described in detail in Section 2.2.7 and 2.2.8.

Assessment of germination

Germination media with fully available water and with imposed water-stress conditions were prepared as described in 2.2.6. Germination media were inoculated with blastospores from a treatment by spreading 100 µl blastospore suspension of $5x10^6$ spores ml^{-1}, made up in 10 ml of sterile distilled water, over the surface using a glass rod. Petri plates of the same a_w were sealed in a polyethylene bag and incubated at 25°C. Three 5 mm discs of medium were removed per replicate after 6 h from the medium with freely available water (0.998 a_w) and after 36 h for the medium with imposed water-stress (0.963 a_w). These were stained with lactophenol cotton blue and the percentage germination was assessed under the light microscope as described in Section 2.2.6. The sample times were identified during a preliminary experiment using blastospores from the control treatment. It was observed that on medium with freely available water, 6 h incubation, and on medium with imposed water-stress, 36 h incubation were appropriate to facilitate adequate measurements.

3.2.4 Impact of osmoprotection on endogenous reserves and ecological fitness

In order to test whether the hyposmotic shock that modified blastospores (blastospores with intracellular water potential lower than 0) undergo when they are washed with water results in leakage of endogenous polyols and subsequent loss of ecological fitness, blastospores from the same treatments as described above (Section 3.2.3) were washed with isotonic solutions of PEG 200 equivalent to the a_w of the medium. Thus, 8, 15, 23, and 34% PEG 200 was used to achieve 0.99, 0.98, 0.97 and 0.96 a_w respectively. Determination of polyols was performed as described previously. For germination assessment the same procedure as described in section 3.2.3 was followed, except that re-suspension of the spores was made in 10 ml of sterile isotonic PEG 200 solution of equivalent a_w to that used for washing of the spores.

3.2.5 Nitrogen screening

After optimisation of water-stress and incubation time conditions, further optimisation was undertaken by screening different nitrogen sources provided at different levels. Glucose was the carbohydrate used, because of its low cost and its established effectiveness for good blastopsore production by *M. anisopliae* (Cambell *et al.*, 1983). The various nitrogen sources tested for optimum blastospore production were cornsteep solid (CS), cottonseed flour (CF) (Sigma), yeast extract (YE), beef extract (BE), mycological peptone (MP) (LAB M), soy protein and KNO_3 (Sigma), all provided at 2, 4 or 7%. The a_w of all media was adjusted to 0.98 by adding 7% PEG 200 (Sigma) + 1% NaCl (Sigma) when the nitrogen source was provided at 2%, 7% PEG 200 + 0.5 % NaCl when the nitrogen source was provided at 4% and 7% PEG 200 + traces of NaCl when the nitrogen source was provided at 2% in either distilled water or basal medium (*Minerals*: KH_2PO_4, 2.0 g; $CaCl_2{\cdot}2H_20$, 0.4 g; $MgSO_4{\cdot}7H_20$, 0.3 g; *Trace metals*: $CoCl_2{\cdot}6H_20$, 37 mg: $FeSO_4{\cdot}7H_20$, 50 mg; $MnSO_4{\cdot}H_20$, 16 mg; $ZnSO_4{\cdot}7H_20$, 14 mg). After addition of the a_w-modifying solutes, final volumes were measured and 2, 4 or 7% of a nitrogen source together with 4% glucose were added. It should be noted that when either CS or CF was used as the nitrogen source, these were added first without the addition of glucose and autoclaved for 30 mins. After that, this was filtered through 2 layers of lens cleaning tissue. In the filtrate, 4% glucose was added. This procedure was

followed because these two nitrogen sources are only entirely soluble after heating. The a_w of all media was measured before autoclaving. The pH of the media was adjusted to 6.8 before autoclaving, using 40% aqueous NaOH. Each treatment was carried out in triplicate 250 ml baffled flasks each containing 50 ml of liquid medium. Flasks containing the liquid medium were autoclaved at 120°C for 15 mins and each one inoculated with 0.5 ml conidial suspension of $5x10^6$ conidia ml^{-1}. Conidia were collected from 10 to 14-day-old cultures grown on MEA at 25°C. Cultures were incubated at 25°C on a rotary shaker at 180 r.p.m. Blastospore counts were done after 72 h of incubation by filtering 1 ml of subsample from each replicate flask through one layer of Lens tissue. Final dry biomass (72 h) determination was also carried out. For this reason 35 ml of liquid culture were centrifuged down for 15 mins at 3000 r.p.m., after which the supernatant was discarded, and the resultant pellet was washed with 35 ml of water and re-centrifuged for 15 mins at 3000 r.p.m. After removal of the supernatant samples were placed in an oven set at 70°C and left for 24 h.

3.2.6 Impact of nitrogen source, a_w-modifying solute and pH on *M. anisopliae* blastospore production, and ecological fitness

Media preparation

Cornsteep solid and CF were found to be the best nitrogen sources for optimum blastospore yield when they were provided at 7%. However, neither of these 2 nitrogen sources gave as high blastospore production when compared to 3% CS + 4 % YE, modified to 0.98 a_w using PEG 200, during the water-stress optimization step. It was decided therefore that either CS (3%) or CF (3%) in combination with YE (4%) as the nitrogen source should be used for the following optimization step. Glucose (4%) was the only carbohydrate used. All media were adjusted to 0.98 a_w using either 7% PEG 200, 2.24% KCl or 1.5% NaCl in distilled water. When either basal medium or distilled water was used in the nitrogen screening step, blastospore production was similar. Cornsteep solid and CF were added after determination of final volumes of modified distilled water and autoclaved for 30 mins. After filtration through two layers of lens tissue, 4% yeast extract and 4% glucose were added to the filtrate. The pH was adjusted to 3.5, 5, 6.8, 8, 9 or 10 using either HCl (5 N) or NaOH (40%). The resultant combinations of medium modifications are shown in Table 3.1.

Table 3.1. Combinations of medium modification using 3 nitrogen sources, 3 a_w-modifying solutes and 5 different pH values.

Nitrogen source	a_w - modifying solute	pH[a]
CS + YE	PEG 200	3.5-10
CS + YE	KCl	3.5-10
CS + YE	NaCl	3.5-10
CF + YE	PEG 200	3.5-10
CF + YE	KCl	3.5-10
CF + YE	NaCl	3.5-10

CS: Cornsteep solid (3%), CF: cottonseed flour (3%), YE: Yeast extract (4%)
[a]The pH values tested were 3.5, 5, 6.8, 8, 9 and 10

Culture conditions and blastospore harvest

Each treatment was carried out in triplicate 250 ml baffled flasks containing 50 ml of liquid medium. Flasks containing the liquid medium were autoclaved at 120°C for 15 mins and each one inoculated with 0.5 ml conidial suspension of $5x10^6$ conidia ml^{-1}. Conidia were collected from 10 to 14-day-old cultures grown on MEA at 25°C. Cultures were incubated at 25°C on a rotary shaker at 180 r.p.m. Blastospores were quantified and harvested at 72 h incubation. The content from a replicate flask was filtered through two layers of sterile lens tissue. The filtrate was centrifuged at 2700 r.p.m for 10 min. After centrifugation the supernatant was used to determine the pH and then discarded. The resultant spore pellet was washed with 10 ml of sterile 15% PEG 200 solution which was isotonic to the medium (0.98 a_w) and re-centrifuged. The supernatant was then discarded and the blastospores were used for intracellular polyol and protein determination, and for germination studies.

Final dry biomass (72 h) determination was also carried out as described in Section 3.2.5.

Extraction and detection of polyols

Twenty to 50 mg of blastospores (fresh weight) from each treatment were used for polyol extraction. The method for polyol extraction and quantification has been described in detail in Section 2.2.7 and 2.2.8.

Total protein content determination

Twenty to 50 mg of blastospores (fresh weight) from each treatment were put into a 2 ml microfuge tube containing 1 ml AnalaR water and sonicated for 60 s at an amplitude of 28 μm using a Fisons Soniprep 150, fitted with a 3.5 mm diameter exponential probe. After sonication samples were centrifuged for 10 mins at 12000 r.p.m. in a microcentrifuge and the supenatant was used for protein determination. Total protein was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma, UK).

Assessment of germination

Assessment of germination of different treatments was done as described in 3.2.3 but only on medium with imposed water-stress because no differences were observed on medium with fully available water in previous studies.

3.2.7 Statistical analysis

As described in Section 2.2.9

3.3 RESULTS

3.3.1 The impact of conidial inoculum size on blastospore production by *M. anisopliae*

Inoculum size studies showed that even a two-fold magnitude difference in the initial conidial inoculum can have a significant impact on final blastospore production by *M. anisopliae* in liquid fermentation (Table 3.2). The lowest threshold of conidial inoculum for blastospore formation was found to be $1x10^6$ conidia ml^{-1}. Below that, the fungus grew in pellet form. Blastospore production increased with increasing inoculum size up to $5x10^6$ conidia ml^{-1}, while further increase in inoculum size resulted in a reduction in blastospore production. Highest blastospore production occurred after 72 h incubation for all inoculum sizes, except for the lowest necessary for blastospore formation ($1x10^6$), where highest blastospore production occurred at 96 h incubation.

Table 3.2. The impact of conidial inoculum size on blastospore production by *M. anisopliae*.

Inoculum size (conidia ml^{-1})	Number of blastospores ml^{-1}			
	Time (h)...			
	48	72	96	120
5×10^7	1.90×10^6	2.01×10^6 a	1.90×10^6 a	++
2×10^7	0	3.37×10^6 b	3.27×10^6 b	++
1×10^7	0	6.86×10^6 c	6.72×10^6 c	++
5×10^6	0	9.93×10^6 d	9.65×10^6 d	++
2×10^6	0	8.72×10^6 d	8.57×10^6 e	++
1×10^6	0	6.48×10^6 c	6.87×10^6 f	6.64×10^6
5×10^5	0	+	+	+

Different letters within a column indicate statistical differences ($P<0.05$) between treatments
+ Pellet formation
++ Cultures were too viscous to allow blastospore harvest

3.3.2 Temporal blastospore production by *M. anisopliae* under different a_w levels and a_w-modifying solutes

Water activity level, a_w-modifying solute and culture age highly influenced blastospore production by *M. anisopliae*. The results from this study are shown in Figure 3.1. Apart from the treatments modified to 0.97 and 0.96 a_w using PEG 200, all other modified treatments gave significantly ($P<0.05$) higher maximum blastospore yield compared to the unmodified treatment (control). Regardless of the a_w- modifying solute, increasing the level of water-stress reduced maximum blastospore yield while extending the incubation time when maximum yield occurred. Optimum blastospore yield occurred in the treatment modified to 0.98 a_w using PEG 200 followed by that modified to the same a_w using KCl or NaCl. Maximum blastospore production at 0.98 a_w level occurred after 72 h incubation, at 0.97 a_w after 84 h incubation and at 0.96 a_w after 120 h incubation. Table 3.3 shows the pH of the spent medium after harvest of blastospores for the incubation times at which studies for polyol accumulation and germinability for each treatment were determined. It should be noted that unmodified cultures, and those modified to 0.98 and 0.97 a_w were too viscous to allow blastospore harvest for germination and HPLC studies after 84 and 120 h incubation respectively.

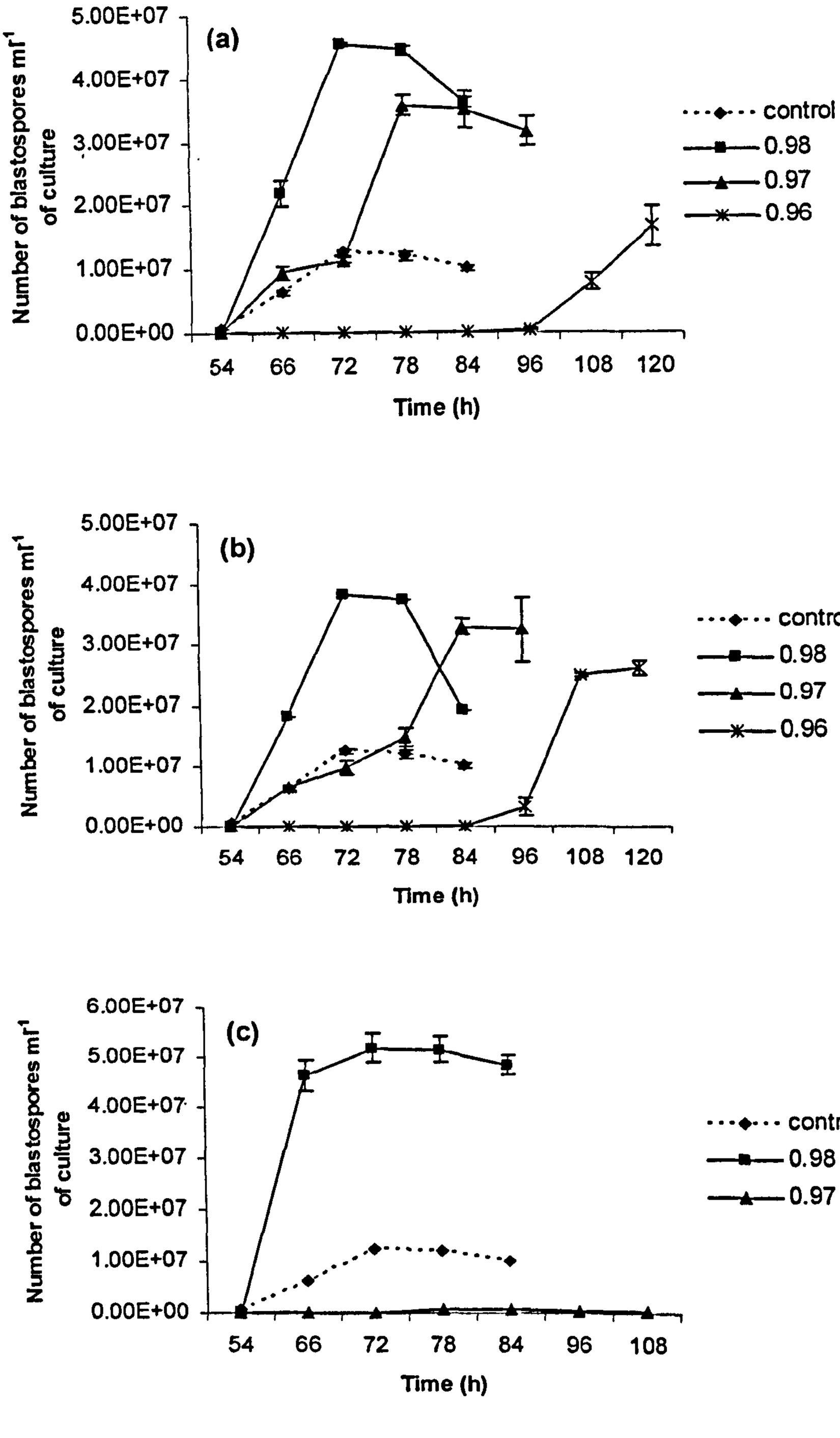


Figure 3.1. Temporal blastospore production of *M. anisopliae* in unmodified (control) and modified with either (a) KCl, (b) NaCl or (c) PEG 200 to 0.98, 0.97 or 0.96 a_w Adámek's medium. No blastospore production occurred when the medium was modified to 0.96 a_w with PEG 200 (c). All data are means of three replicates per treatment. Bars represent standard errors of the means.

Table 3.3. Values of pH (± standard error) of spent unmodified and modified Adámek's medium after harvest of *M. anisopliae* blastospores in relation to a_w and incubation time.

	pH of spent medium					
	a_w	**0.98**		**0.97**		**0.96**
Modifying solute	**Time (h)**					
	72	**84**	**72**	**84**	**108**	**120**
Unmodified[+]	4.61±0.01*					
KCl	4.52±0.01*	4.52±0.01	4.92±0.06	4.63±0.06*	5.46±0.02	4.82±0.01*
NaCl	4.42±0.05*	4.49±0.08	4.87±0.06	4.43±0.02*	5.04±0.02	4.61±0.01*
PEG 200	5.18±0.05*	5.16±0.03	5.77±0.02	5.72±0.01*	5.95±0.01	5.92±0.01

+ a_w = 0.989
* indicates the time for each a_w level when optimum spore production occurred.

3.3.3 Endogenous reserves in *M. anisopliae* blastospores in relation to washing treatment, water-stress, and culture age

The a_w-modifying solute PEG 200 was not included in the study of endogenous polyol accumulation, because it impaired blastospore production when a_w modification was adjusted to lower than 0.98. Very low amounts (< 3 mg g^{-1} of fresh weight) of arabitol were detected in blastospores from all treatments and for either post-harvest washing treatment (water or isotonic PEG 200) (data not shown). Glycerol and trehalose were completely absent from all treatments. Figure 3.2 shows the effect of post-harvest washing treatment on the retention of accumulated endogenous erythritol in blastospores of *M. anispoliae* produced under different a_w levels and at different times. It is shown that regardless of a_w level, a_w-modifying solute or culture age, higher amounts of erythritol were retained in blastospores that were washed with isotonic PEG 200 solution compared to those that were washed with water. The most remarkable difference between the two post-harvest washing treatments was observed in blastospores produced under high water-stress (0.96 a_w) followed by blastospores produced at intermediate water-stress (0.97 a_w) and after 84 h incubation. It is interesting to note that no difference in endogenous erythritol retention between the two post-harvest washing treatments was observed after 72 h incubation for blastospores

produced at intermediate water-stress (0.97 a_w) and in the control treatment. Another interesting observation is that regardless of post-harvest washing treatment, endogenous erythritol concentration increased with culture age (no comparison can be made for treatments modified at 0.96 a_w, as not enough spores were produced from these treatments at the first sampling time (108 h) to allow HPLC analysis).

The effect of post-harvest washing treatment on the retention of endogenous amounts of the high molecular weight polyol mannitol is shown in Figure 3.3. In this case post-harvest washing with isotonic PEG 200 solution resulted in a significant ($P<0.05$) polyol retention in blastospores produced after 72 h and at 0.98 a_w (for either a_w-modifying solute) and in blastospores produced after 84 h and intermediate water stress (0.97 a_w), compared to that washed with water. No effect on retention of endogenous mannitol between the two post-harvest washing treatments was observed in blastospores produced under high water-stress conditions (0.96 a_w). Again, as in the case of erythritol, endogenous mannitol concentration increased with culture age.

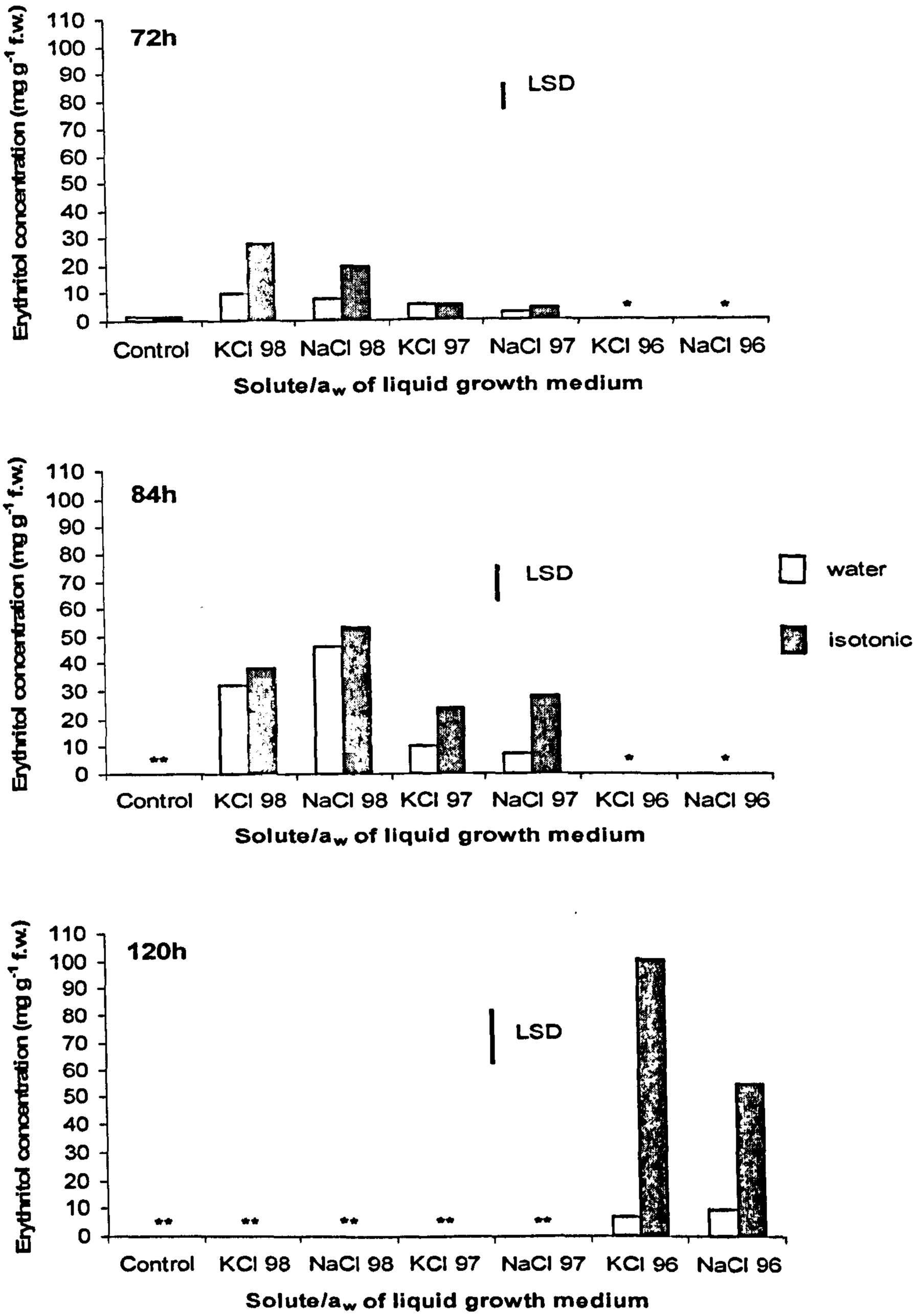
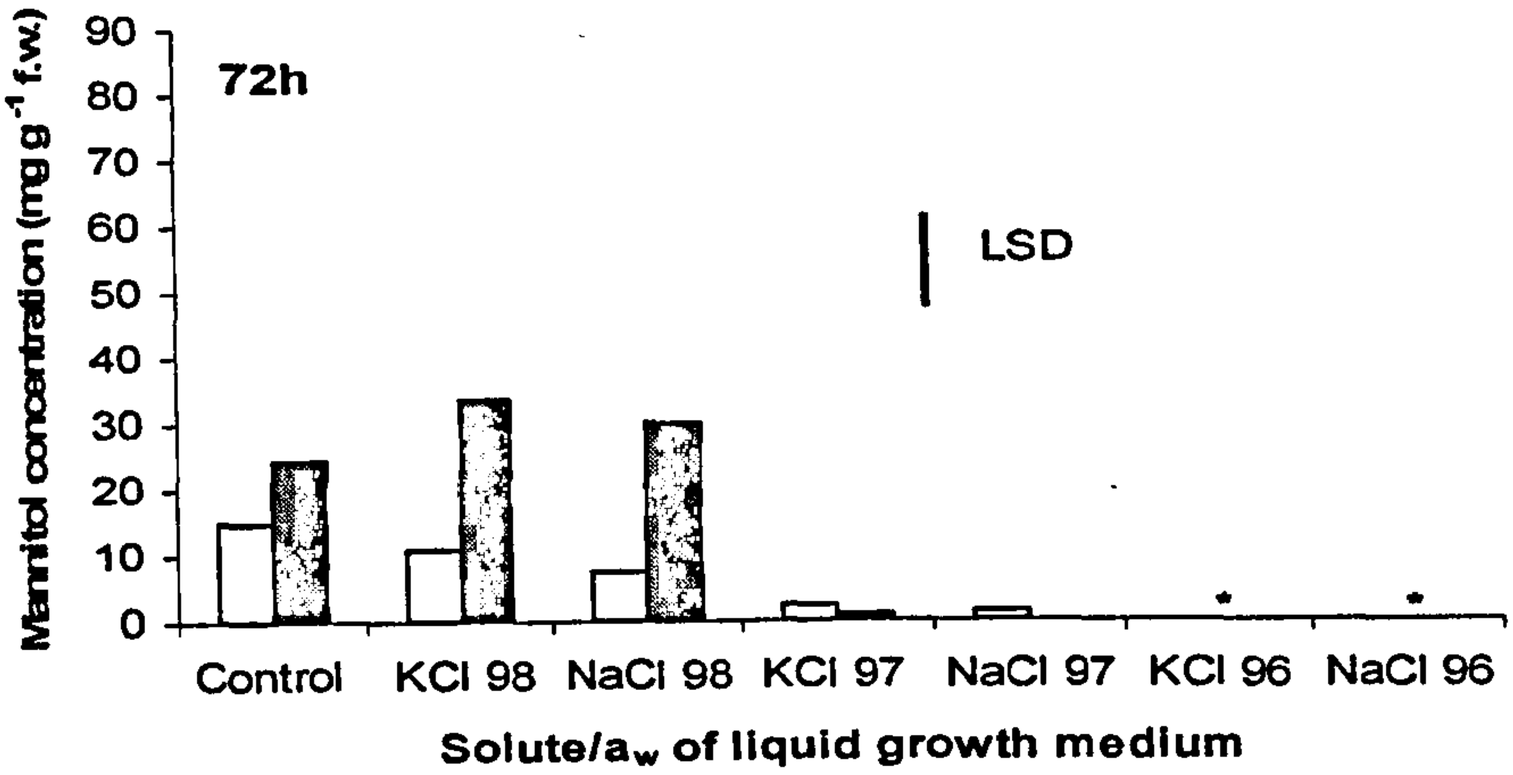
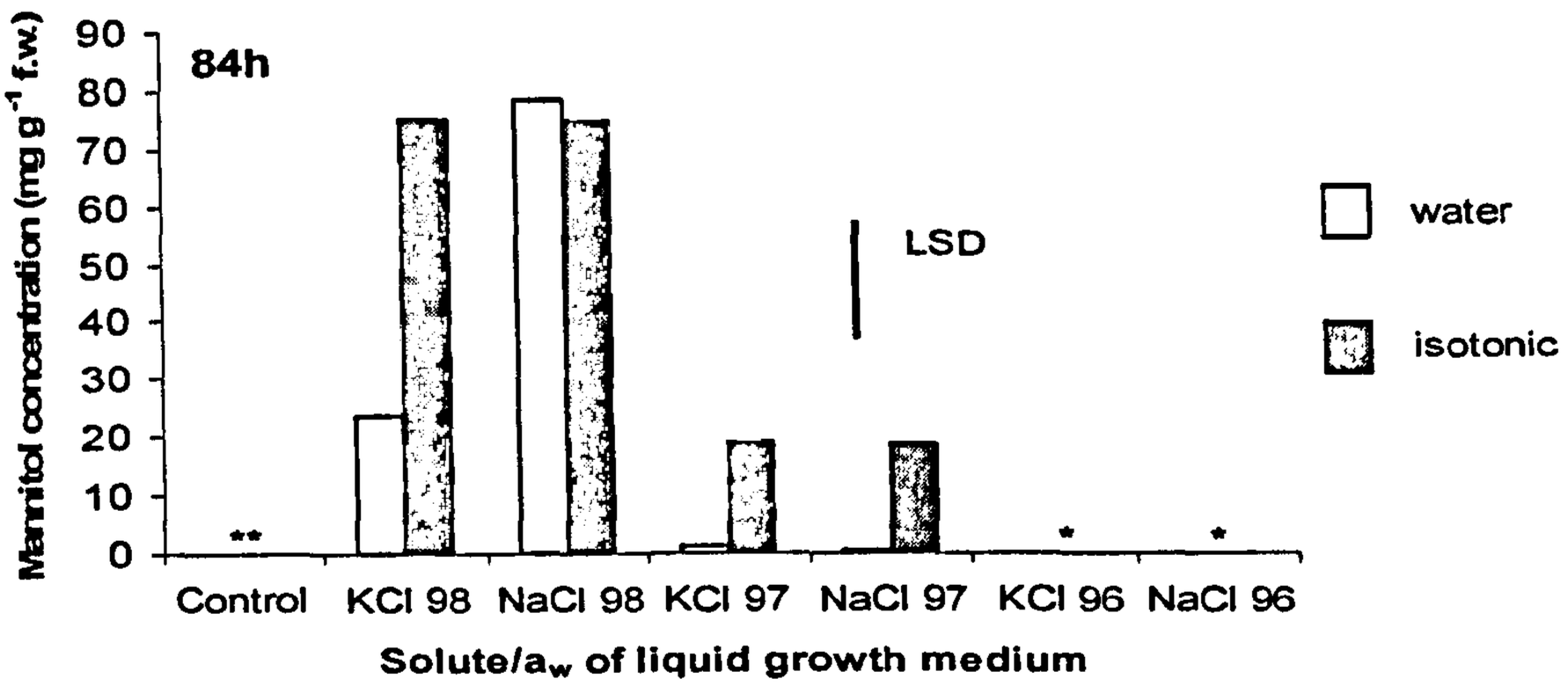


Figure 3.2. The effect of post-harvest washing treatment on the retention of endogenous erythritol in blastospores of *M. anisopliae* produced in unmodified (control) and modified with either KCl or NaCl to 0.98, 0.97 or 0.96 a_w and at different incubation times. All data are means of three replicates per treatment. Bars indicate least significant differences (LSD) ($P<0.05$) of the means.

* No blastospore production

** Cultures were too thick to allow blastospore harvest





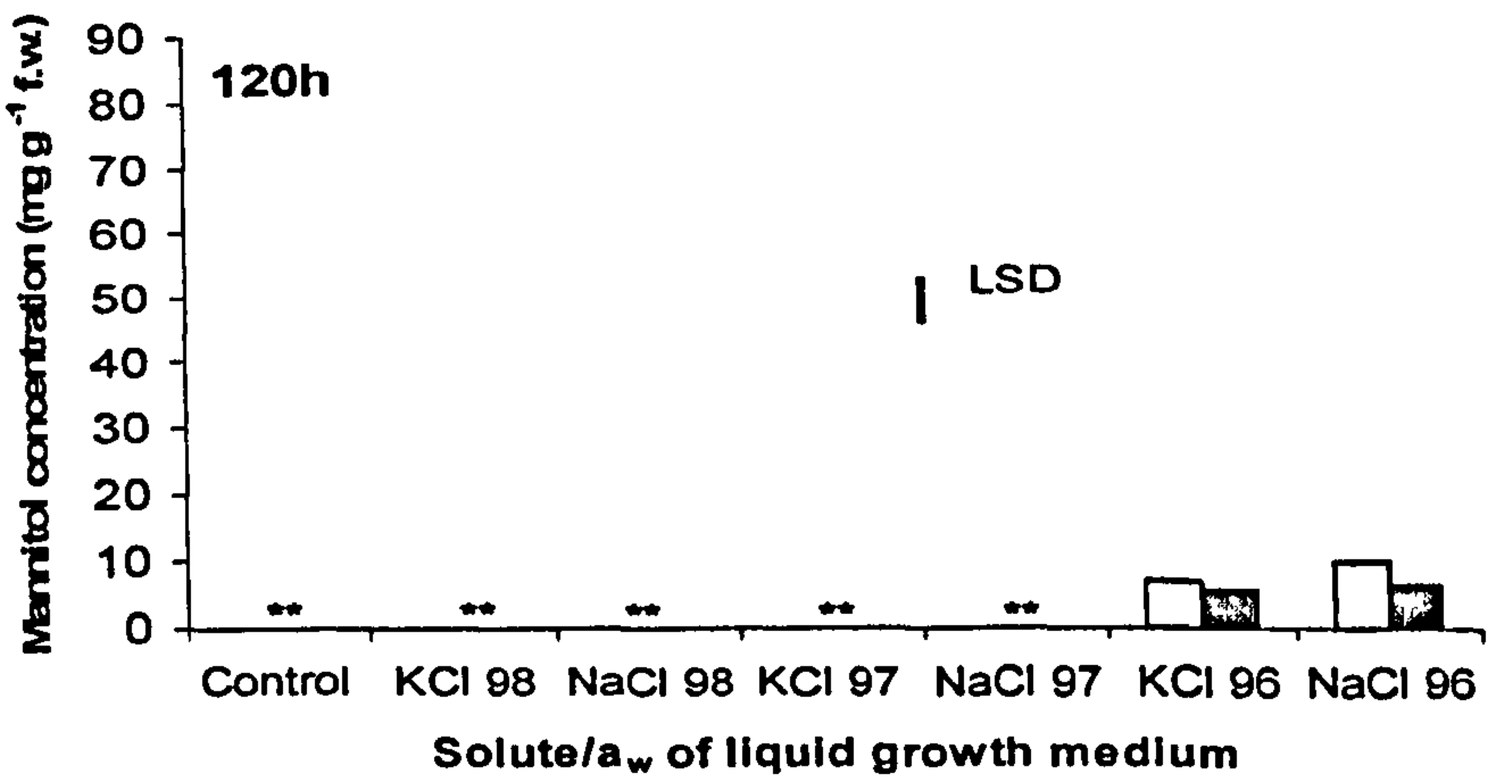


Figure 3.3. The effect of post-harvest washing treatment on the retention of endogenous mannitol in blastospores of *M. anisopliae* produced in unmodified (control) and modified with either KCl or NaCl to 0.98, 0.97 or 0.96 a_w and at different incubation times. All data are means of three replicates per treatment. Bars indicate least significant differences (LSD) ($P<0.05$) of the means.

* No blastospore production

** Cultures were too thick to allow blastospore harvest

3.3.4 Loss of endogenous reserves of blastospores during hypo-osmotic shock

Table 3.4 shows the amounts of the compounds investigated in this study that were detected in the water used for washing the blastospores before they were submitted to polyol analysis. Remarkably higher amounts of the low molecular weight erythritol were detected in the water from blastospores produced in the modified media compared to blastospores from the unmodified medium. This difference was not significant ($P<0.05$) with blastospores from the 72-h-cultures modified to 0.97 a_w. The trend was different with the high molecular weight polyol mannitol, where only washings from blastospores produced in the 0.98 a_w modified treatment contained higher amounts of this compound compared to blastospores from the unmodified medium. No trehalose or glycerol was detected in the washings of any treatment.

Table 3.4. Amounts of solutes (ppm) detected in the water used to wash the blastopores after harvest. All data are means of three replicates per treatment. Least significant differences (LSD) ($P<0.05$) between values from different treatments are shown for each solute.

Solute/a_w	Time (h)	Erythritol	Arabitol	Mannitol
Control	**72**	36.93	27.05	26.12
KCl 0.98	**72**	254.63	18.25	93.28
	84	59.43	20.31	42.14
NaCl 0.98	**72**	309.97	17.91	134.13
	84	486.83	22.76	68.3
KCl 0.97	**72**	43.81	2.17	2.04
	84	347.53	26.69	22.43
NaCl 0.97	**72**	59.091	7.03	1.56
	84	289.5	31.64	3.75
KCl 0.96	**108**	*	*	*
	120	392.1	43.43	4.49
NaCl 0.96	**108**	*	*	*
	120	352.6	40.37	9.35
LSD		151.2	13.8	27.45

* indicates not enough blastospore production for HPLC analysis.

3.3.5 Germination efficiency of modified *M. anisopliae* blastospores in relation to washing treatment and culture age

When germination was tested on water agar medium with freely available water, no difference was observed between any of the treatments, regardless of post-harvest washing with either water or isotonic PEG 200 solution. All treatments had a high percentage germination (> 90%) (data not shown). However, when germination was tested on water agar medium with imposed water-stress (0.96 a_w) there were remarkable differences between treatments (Figure 3.4). Post-harvest washing with isotonic PEG 200 solution resulted in a significant ($P<0.05$) increase in germinability for blastospores produced under unmodified and that modified to 0.98 a_w conditions compared to washing with water. However, post-harvest isotonic washing treatments did not confer any difference in germinability for blastospores produced under 0.97 a_w, and it reduced germinability of blastospores produced under the highest water-stress treatment (0.96 a_w) compared to washing with water. It is interesting to note that blastospores produced at 0.97 a_w after 72 h incubation regardless of washing treatment, did not germinate at all on water-stressed agar medium (Figure 3.4), whereas germination was very high (90%) on agar with freely available water (data not shown).

Culture age also had an effect on blastospore germinability. For all a_w treatments when blastospores were washed with water, germinability increased with culture age. When blastospores were washed with isotonic PEG 200 solution, germinability increased with culture age only for blastospores produced at 0.98 a_w.

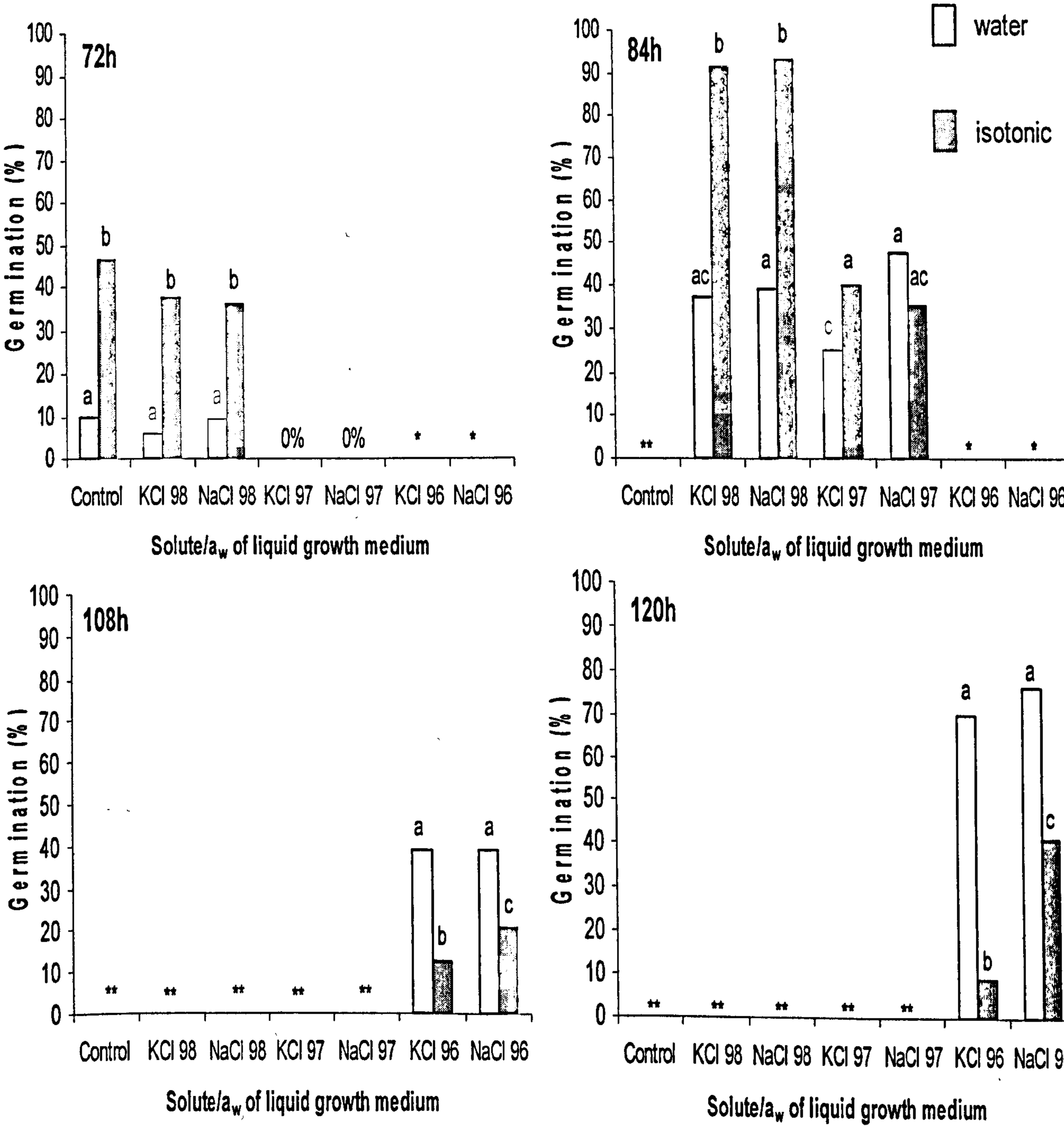


Figure 3.4. Germination (%) of *M. anisopliae* blastospores produced in unmodified (control) and modified with either KCl or NaCl to 0.98, 0.97 or 0.96 a_w and at different incubation times, after 36 h incubation at 25°C and on water agar modified to 0.96 a_w with PEG 200. All data are means of three replicates per treatment. Different letters between two treatments indicate significant differences ($P<0.05$).

* No blastospore production

** Cultures were too thick to allow blastospore harvest

3.3.6 The impact of nitrogen source on blastospore production by *M. anisopliae*

Blastospore production by *M. anisopliae* was highly influenced by different nitrogen sources (Table 3.5), ranging from no blastospore induction to production of high numbers of blastospores (> 10^7 blastospores ml^{-1} of culture). For all nitrogen sources tested, blastospore production increased with increasing nitrogen source concentration (from 2 to 7%).

Final dry biomass also increased with increasing nitrogen source concentration, except for YE and soy protein where final dry biomass increased from 2 to 4% nitrogen source concentration and then declined. An increase in final biomass resulted in a significant ($P<0.05$) increase in blastospore production for the 3 best nitrogen sources (CS, CF, BE) but not for MP. It is interesting to note that the presence of organic nitrogen was essential for filamentous growth of the fungus, as when only inorganic nitrogen was provided, the fungus grew in pellets.

Table 3.5. Effect of nitrogen source and concentration on *M. anisopliae* blastospore yield and final dry biomass after 72 h of incubation[a]

Nitrogen source	Nitrogen source concentration (%)	Blastospore yield (spores $ml^{-1} \pm SE^b$)	Final dry biomass ($mg\ ml^{-1} \pm SE$)	Culture morphology
Cornsteep solid	2	$4.4 \times 10^6 \pm 8.91 \times 10^5$ a	8.4 ± 0.1 a	Filamentous
	4	$1.4 \times 10^7 \pm 3.79 \times 10^5$ b	12.1 ± 0.3 a	Filamentous
	7	$1.9 \times 10^7 \pm 3.84 \times 10^5$ c	15.3 ± 2.9 b	Filamentous
Cottonseed flour	2	$1.4 \times 10^7 \pm 7.37 \times 10^5$ a	3.8 ± 0.3 a	Filamentous
	4	$1.9 \times 10^7 \pm 6.66 \times 10^5$ b	4.2 ± 0.1 a	Filamentous
	7	$4.2 \times 10^7 \pm 2.49 \times 10^6$ c	14.9 ± 0.8 b	Filamentous
Beef extract	2	$7.5 \times 10^5 \pm 1.33 \times 10^5$ a	10.8 ± 0.2 a	Filamentous
	4	$8.5 \times 10^6 \pm 1.56 \times 10^6$ b	11.2 ± 0.5 a	Filamentous
	7	$1.8 \times 10^7 \pm 3.46 \times 10^5$ c	13.4 ± 0.2 b	Filamentous
Yeast extract	2	$1.5 \times 10^6 \pm 3.48 \times 10^4$ a	6.5 ± 0.1 a	Filamentous
	4	$3.0 \times 10^6 \pm 4.28 \times 10^5$ b	6.8 ± 0.4 a	Filamentous
	7	$3.3 \times 10^6 \pm 3.25 \times 10^5$ b	5.5 ± 0.1 b	Filamentous
Mycological peptone	2	$4.8 \times 10^6 \pm 8.81 \times 10^5$ a	5.8 ± 0.2 a	Filamentous
	4	$5.0 \times 10^6 \pm 6.77 \times 10^5$ a	22.7 ± 6.5 b	Filamentous
	7	$5.1 \times 10^6 \pm 7.38 \times 10^5$ a	41.3 ± 3.4 c	Filamentous
Soy protein	2	0	1.7 ± 0.2 a	Filamentous
	4	0	5.4 ± 0.2 b	Filamentous
	7	0	0.9 ± 0.1 c	Filamentous
KNO_3	2	0	negligible	Pellets
	4	0	negligible	Pellets
	7	0	negligible	Pellets

[a]Cultures were grown in basal salts medium with 4% glucose as the sole carbon source
[b]Standard error
Different letters for each nitrogen source indicate statistical differences ($P<0.05$)

3.3.7 The effect of nitrogen source, modifying solute and pH on *M. anisopliae* blastospore production and growth

Figure 3.5 shows the effect of pH, nitrogen source and a_w-modifying solute on blastospore production and growth of *M. anisopliae*. The ANOVA analysis of the data showed that all three factors (pH, nitrogen source and a_w-modifying solute) individually, as well as the two and three-way interactions between these factors had a very significant effect ($P<0.001$) on blastospore production (Appendix I). Overall, CF was superior to CS in terms of blastospore production under the conditions studied. Under all conditions studied, no growth occurred at 3.5 pH. For all conditions, blastospore production at pH 5 was significantly ($P<0.05$) lower than blastospore production at pH levels between 6.8 and 9 where maximum blastospore yield occurred. At pH 10, either none or very low ($< 2 \times 10^6$) numbers were produced when KCl or NaCl were used as the a_w-modifying solute, but high ($> 3 \times 10^7$) when PEG 200 was used as the a_w-modifying solute.

Nitrogen source and the interaction between nitrogen and pH did not have a significant effect ($P<0.001$) on the growth of *M. anisopliae*, evaluated as final dry biomass. The impact of the other two factors (pH and a_w-modifying solute) and their two way interaction was very significant ($P<0.001$) on *M. anisopliae* growth (Appendix I). Which of the two nitrogen sources supported better growth depended predominantly on the pH and a_w-modifying solute. Overall, for all conditions tested, optimum growth occurred at either pH 6.8 or 8.

Figure 3.6 shows the pooled results of the effect of pH, nitrogen source and a_w-modifying solute on blastospore production and growth of *M. anisopliae*. This representation shows that optimum conditions for maximum blastospore yield occurred at pH values between 6.8 and 8 with the CF+YE as the nitrogen source and KCl as the modifying solute. However, maximum growth occurred between pH 6.8 and 9 with CS + NaCl.

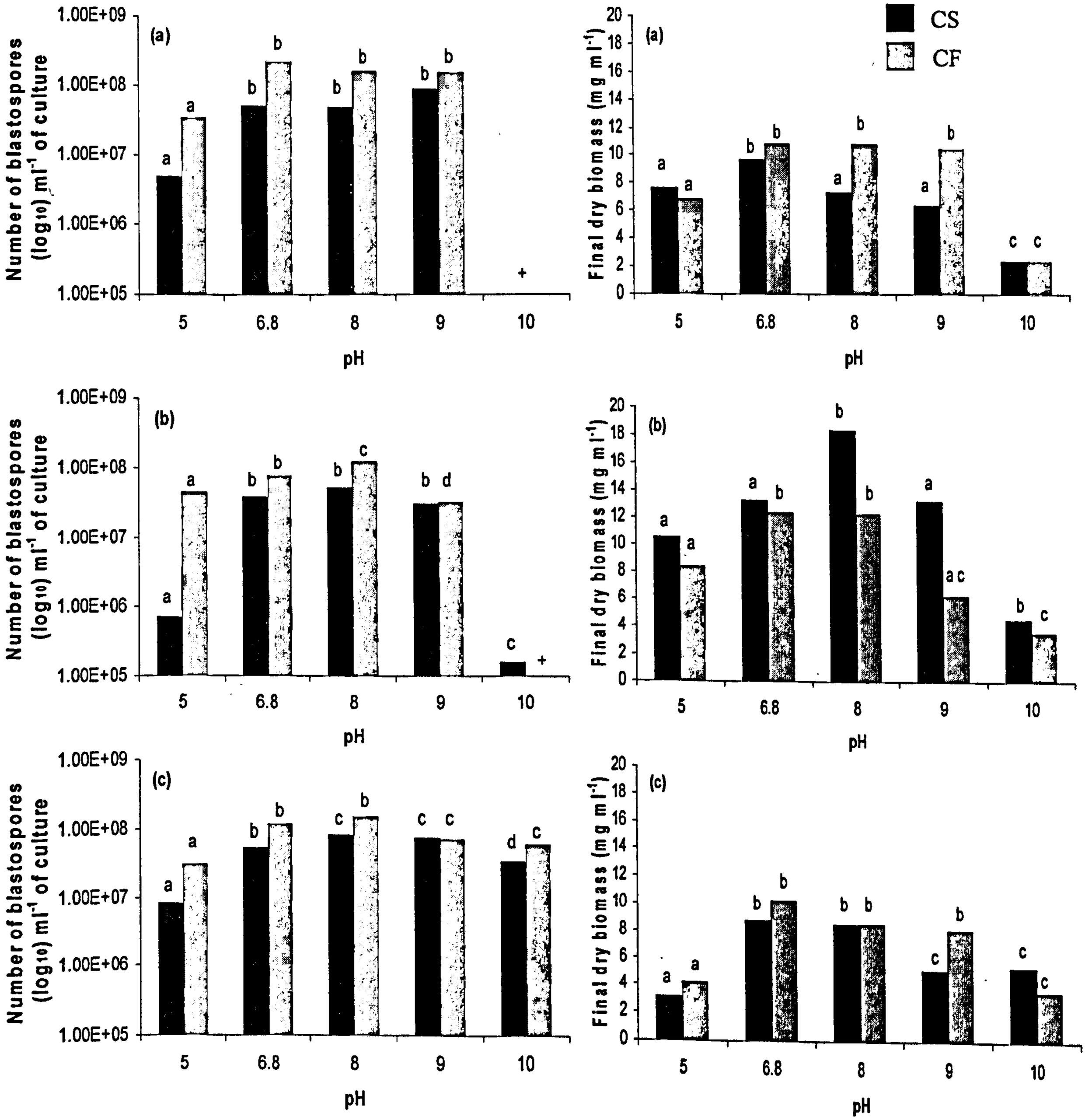


Figure 3.5. The effect of pH, nitrogen source and a_w-modifying solute on blastospore production and growth of *M. anisopliae*. Cultures were grown for 72 h in liquid medium consisting of either 3% CS (cornsteep solid) or 3% CF (cottonseed flour) plus 4% yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either (a) KCl, (b) NaCl or (c) PEG 200. All data are means of three replicates per treatment. Different letters between two means for each nitrogen source indicate significant differences ($P<0.05$).

\+ No blastospore production

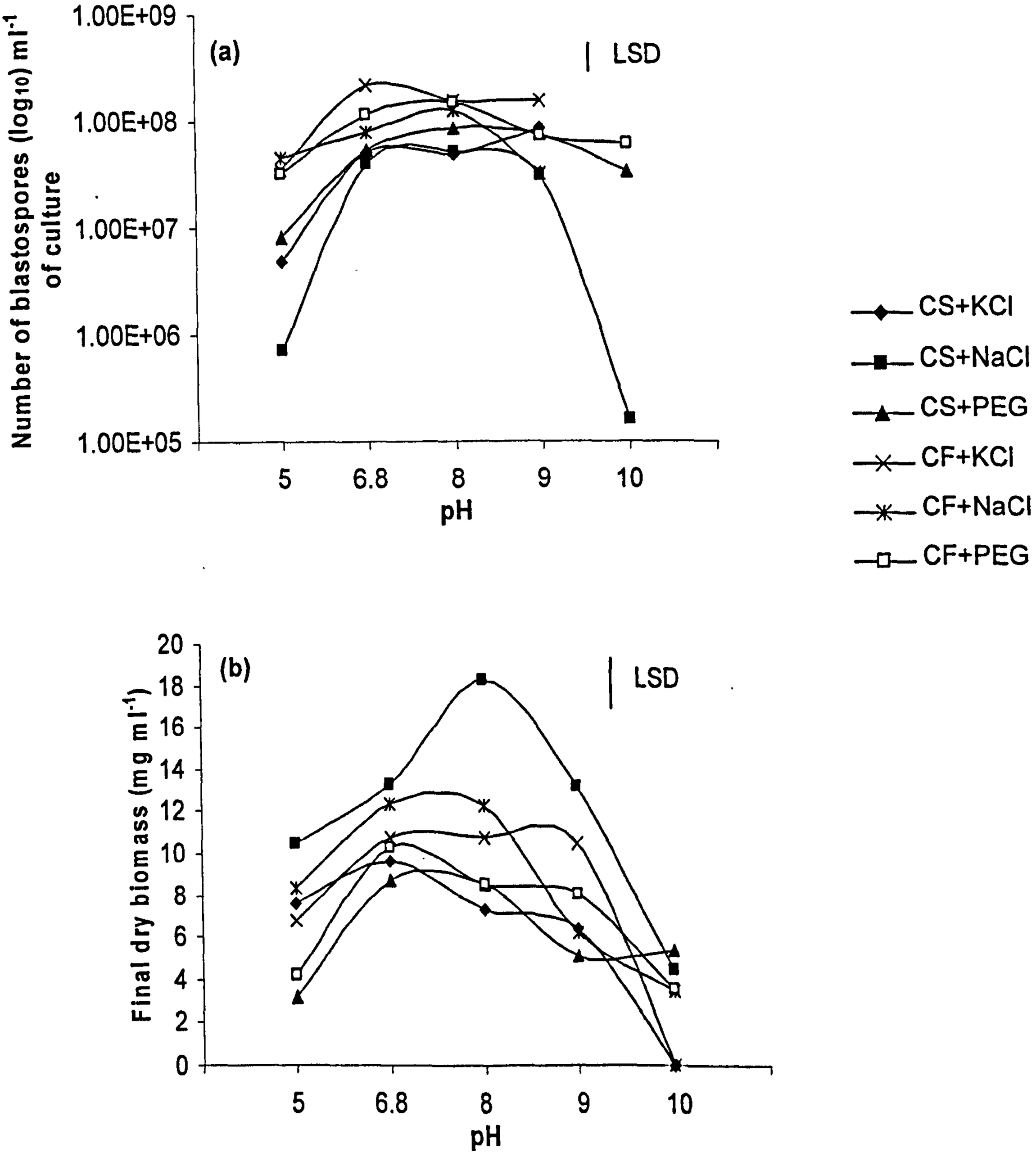


Figure 3.6. Representation of pooled results of the effect of pH, nitrogen source and a_w-modifying solute on blastospore production and growth of *M. anisopliae*. Cultures were grown in liquid medium consisting of either 3% CS (conrnteep solid) or 3% CF (cottonseed flour) plus 4% yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either KCl, NaCl or PEG 200.

Table 3.6. shows the values of pH of spent medium in relation to initial pH, nitrogen source and modifying solute. When growth occurred (pH 5-10) final pH was always lower than the initial pH. No change in the pH was observed when there was no growth (pH 3.5).

Table 3.6. Values of pH of spent medium after harvest of *M. anisopliae* blastospores in relation to initial pH, nitrogen source and modifying solute.

	pH of spent medium					
Nitrogen source+ solute	**Initial pH...**					
	3.5	**5**	**6.8**	**8**	**9**	**10**
CS+KCl	3.51±0.01	4.45±0.02	4.52±0.01	5.01±0.08	5.27±0.31	6.50±0.09
CS+NaCl	3.52±0.01	4.41±0.03	4.42±0.05	4.96±0.08	5.62±0.07	6.43±0.01
CS+PEG	3.51±0.01	4.93±0.01	5.18±0.05	5.50±0.07	5.82±0.03	6.70±0.05
CF+KCl	3.51±0.01	4.33±0.01	4.32±0.01	4.64±0.07	5.23±0.06	6.81±0.02
CF+NaCl	3.53±0.01	4.54±0.02	4.39±0.05	4.89±0.10	5.52±0.06	6.30±0.01
CF+PEG	3.52±0.01	4.88±0.02	5.07±0.05	5.34±0.06	5.69±0.50	6.86±0.04

CS: cornsteep solid; CF: cottonseed flour

3.3.8 The effect of nitrogen source, modifying solute and pH on endogenous reserves of polyols and protein in *M. anisopliae* blastospores

Figure 3.7 shows the effect of pH, nitrogen source and a_w-modifying solute on endogenous polyol accumulation in *M. anisopliae* blastospores. The ANOVA analysis of the data showed that all three factors (pH, nitrogen source and a_w-modifying solute) individually, as well as the two and three-way interactions between these factors had a significant effect ($P<0.05$) and a very significant effect ($P<0.001$) on erythritol and mannitol concentration respectively (Appendix I). In the case of arabitol only pH and modifying solute as well as their interaction had a significant effect ($P<0.05$). The prevailing polyols for all conditions tested were the lower molecular weight erythritol and the high molecular weight mannitol. Glycerol was not detected in any of the treatments. Arabitol was present in very low amounts (< 2.5 mg g^{-1} f.w.). For all treatments, erythritol concentration increased with increasing pH up to a value of 8 except for the CF+KCl treatment and where mannitol concentration was higher than erythritol concentration. Overall, higher concentrations of erythritol were obtained with the CF+YE nitrogen source compared to CS+YE. Cultures modified with KCl or NaCl

produced blastospores with higher amounts of erythritol and mannitol compared to cultures modified with PEG 200.

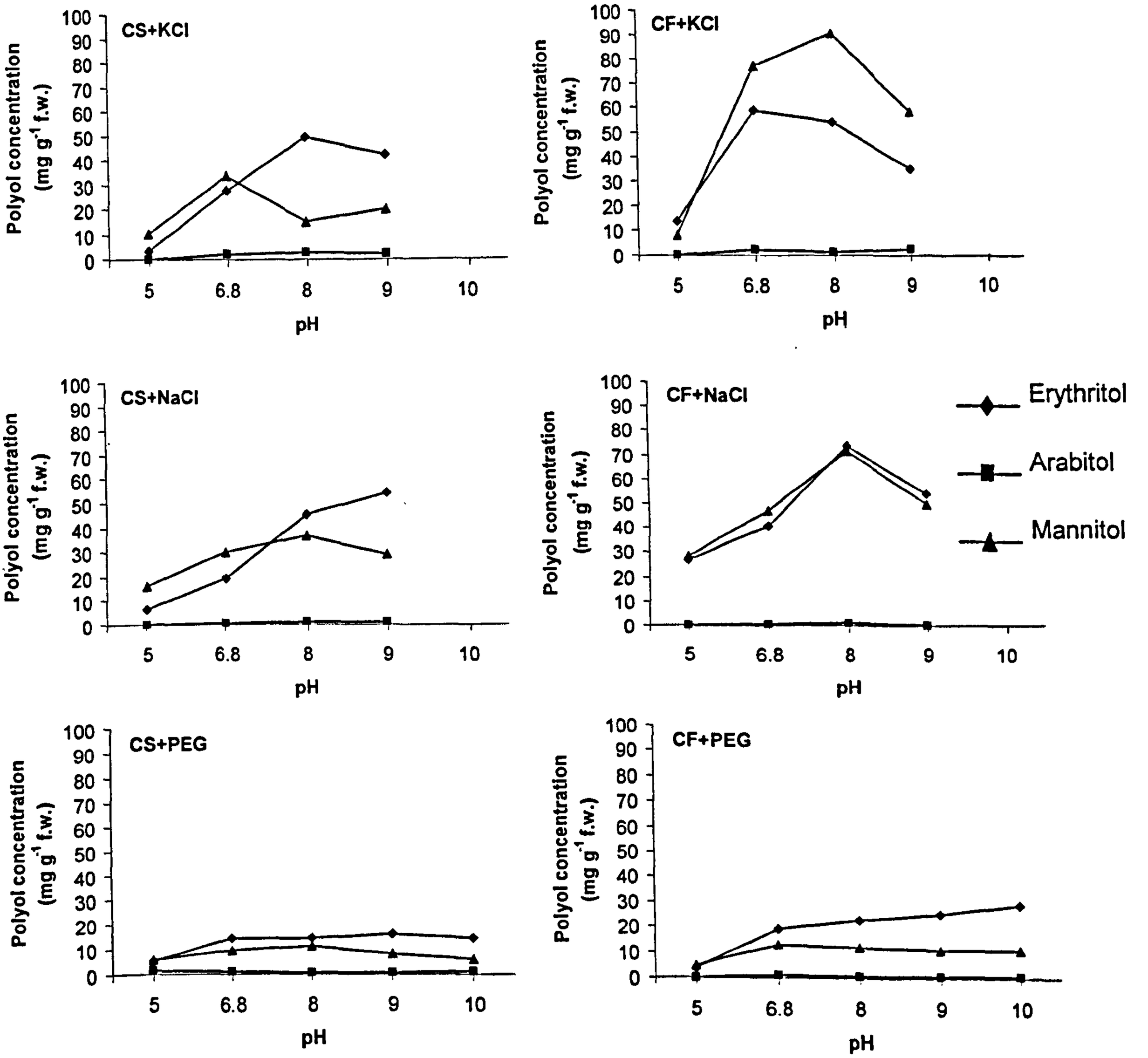


Figure 3.7. The effect of pH, nitrogen source and a_w-modifying solute on endogenous accumulation of polyols in *M. anisopliae* blastospores. Cultures were grown in liquid medium consisting of either 3% CS (cornsteep solid) or 3% CF (cottonseed flour) plus 4% yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either KCl, NaCl or PEG 200. Results are means of three replicates; the least significant differences ($P<0.05$) for erythritol, arabitol and mannitol were: for (CS+KCl) 18.30, 2.22 and 11.26; for (CF+KCl) 20.52, 2.27 and 39.73; for (CS+NaCl) 6.88, 0.52 and 16.52; for (CF+NaCl) 12.94, 0.21 and 12.57; for (CS+PEG) 6.92, 1.70 and 3.78; and for (CF+PEG) 4.23, 0.47 and 3.70 respectively.

The pooled results of the effect of pH, nitrogen source and a_w-modifying solute on endogenous erythritol and mannitol concentration in *M. anisopliae* blastospores are shown in Figure 3.8. Optimum cultural conditions for production of blastospores with maximum endogenous erythritol and mannitol occurred at pH 6.8-8 with the CF+YE as the nitrogen source and either NaCl or KCl as the a_w-modifying solute.

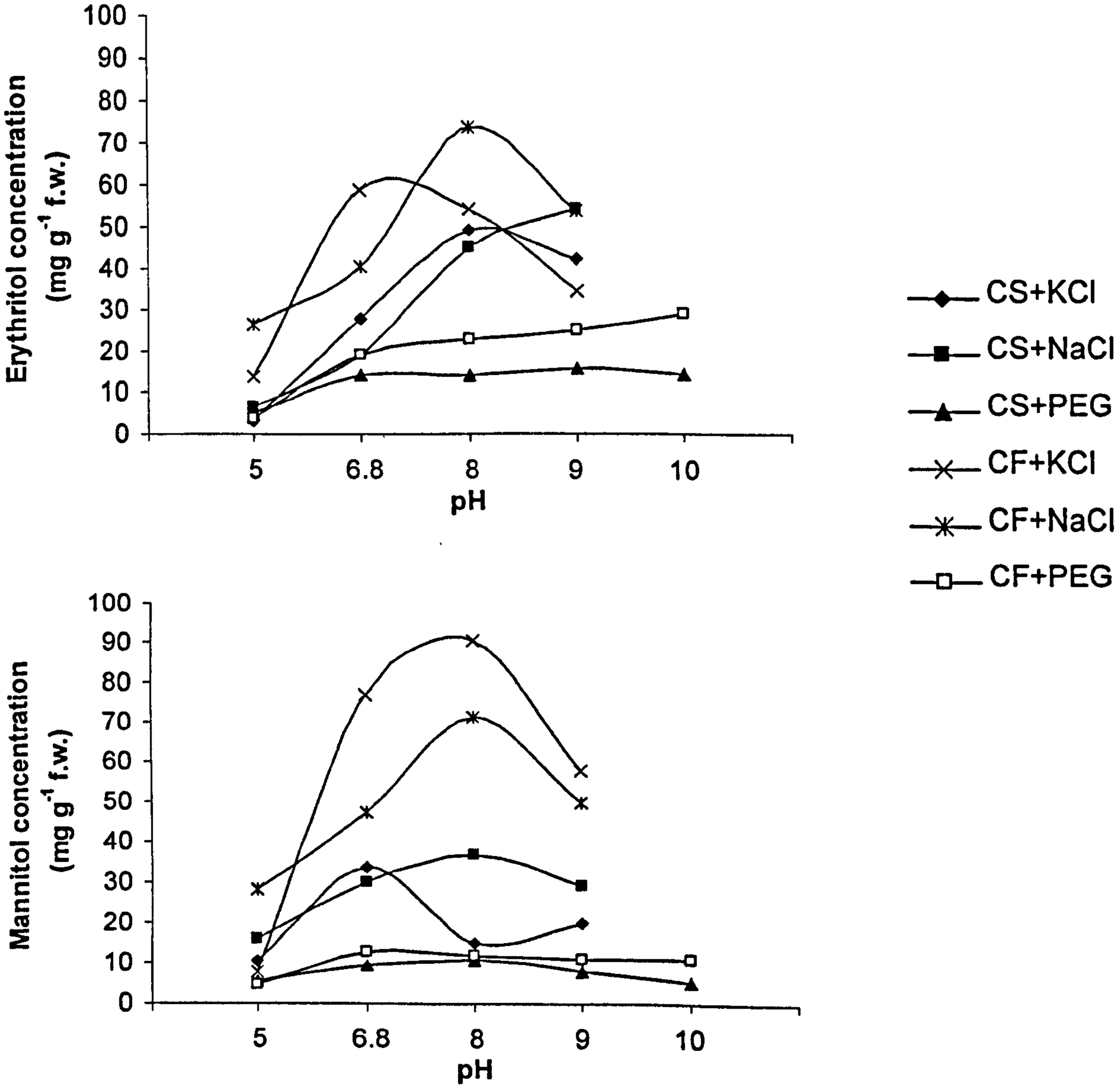


Figure 3.8. Representation of pooled results of the effect of pH, nitrogen source and a_w-modifying solute on endogenous erythritol and mannitol concentration in *M. anisopliae* blastospores. Cultures were grown in liquid medium consisting of either 3% CS (cornsteep solid) or 3% CF (cottonseed flour) plus 4% yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either KCl, NaCl or PEG 200.

Overall, blastospores produced when CF was the additional nitrogen source, contained higher amounts of total protein compared to the ones produced when CS was the additional nitrogen source (Figure 3.9). For both nitrogen sources (CS, CF) and for all three modifying solutes, higher endogenous concentrations of total protein occurred between 6.8 and 8 pH values.

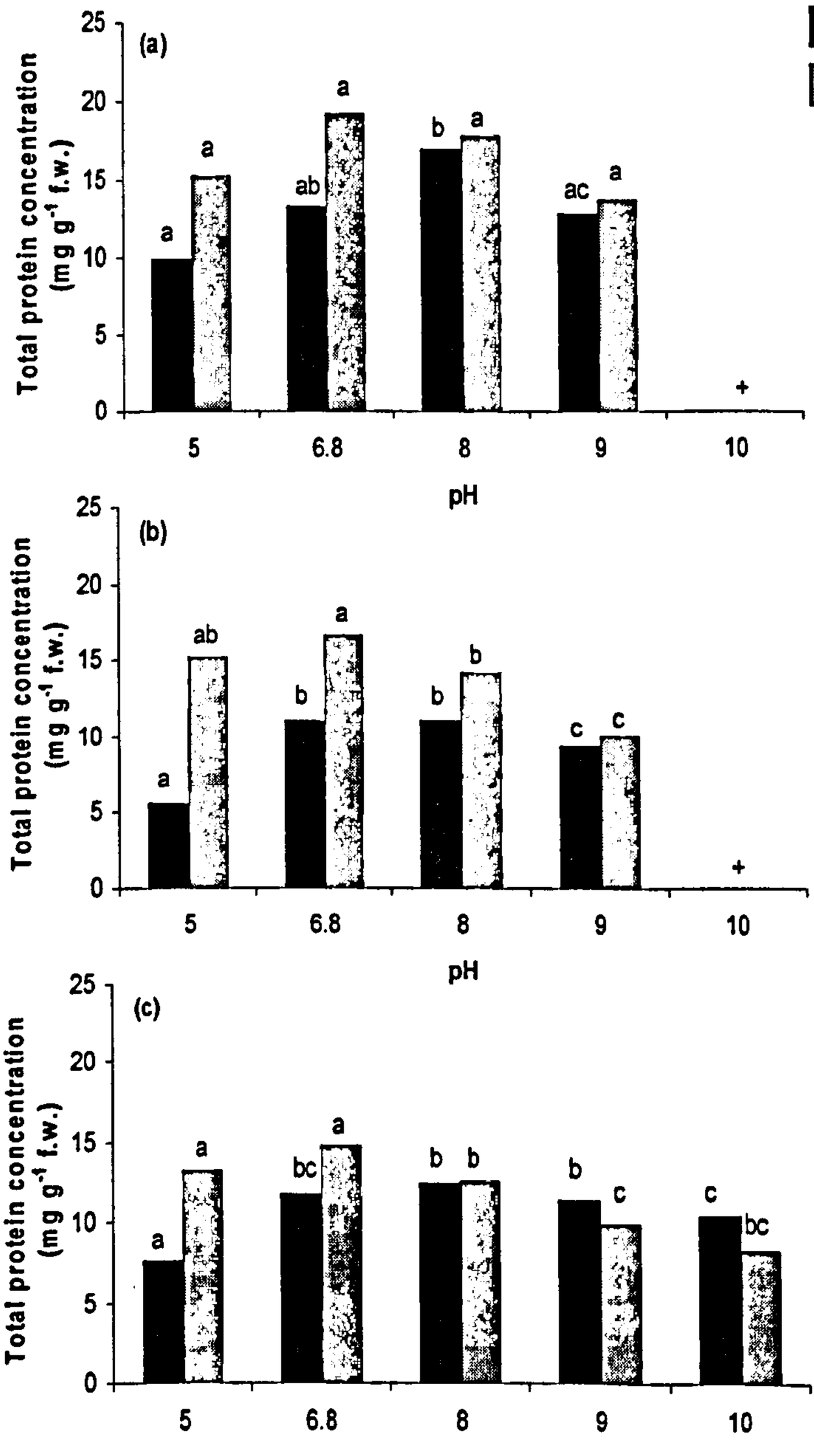


Figure 3.9. The effect of pH, nitrogen source and a_w-modifying solute on endogenous total protein content in blastospores of *M. anisopliae*. Cultures were grown in liquid medium consisting of either 3% CS (cornsteep solid) or 3% CF (cottonseed flour) plus 4% yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either (a) KCl, (b) NaCl or (c) PEG 200. All data are means of three replicates per treatment. Different letters between two means for each nitrogen source (CS, CF) indicate significant differences ($P<0.05$).
+ None or too low blastospore production to enable studies

3.3.9 The effect of nitrogen source, modifying solute and pH on the size of *M. anisopliae* blastospores

The impact of different environmental conditions (nitrogen source, a_w-modifying solute and pH) on blastospore size is shown in Table 3.7. The width of *M. anisopliae* blastospores did not differ markedly between treatments and it ranged between 3-3.5 μm. Regardless of the additional nitrogen source and modifying solute, blastospore length increased with increasing pH. Under the same pH and modifying solute conditions, cultures containing CF+YE as the nitrogen source produced smaller blastospores compared to cultures containing CS+YE, except for CF+KCl/pH 5 and CF+NaCl/pH 6.8.

Table 3.7. Impact of nitrogen source, a_w-modifying solute and pH on blastospores size (± standard error) of *M. anisopliae*.

		Blastospore length (μm)				
Nitrogen source+ solute	**pH...5**	**6.8**	**8**	**9**	**10**
CS+KCl	8.06±0.14	9.64±0.21	11.24±0.09	12.56±0.38	+
CF+KCl	8.14±0.08	8.93±0.20	9.48±0.27	10.44±0.11	+
CS+NaCl	9.13±1.09	9.23±0.10	10.35±0.23	11.22±0.31	11.97±0.65
CF+NaCl	8.08±0.11	9.25±0.08	9.37±0.27	10.20±0.18	+
CS+PEG	8.19±0.04	10.03±0.14	10.58±0.11	12.90±0.78	13.25±0.37
CF+PEG	7.75±0.03	9.03±0.25	9.95±0.10	11.25±0.34	12.05±0.31

CS: cornsteep solid; CF: cottonssed flour

3.3.10 Germination efficiency of characterised *M. anisopliae* blastospores produced under different nitrogen, pH and a_w-modifying solute conditions

Each factor individually as well as their two and three-way interactions had a very significant effect ($P<0.001$) on both the percentage germination and the germ tube length of characterised *M. anisopliae* blastospores (Appendix I). Liquid culture with the CF+YE as the nitrogen source, resulted in the production of blastospores with better germination efficiency (expressed as percentage germination, or germ tube length) compared to cultures with CS+YE as the nitrogen source, under most of the conditions tested (Figure 3.10). Regardless of the additional nitrogen source and a_w-modifying solute, percentage germination was the lowest at pH 5 and optimum between 6.8 and 9.

The pooled results for percentage germination and germ tube length are show in Figure 3.11. Blastospores with very high germination efficiency were produced from CF+KCl cultures between 6.8 and 8 pH followed by CF+NaCl at the same pH range.

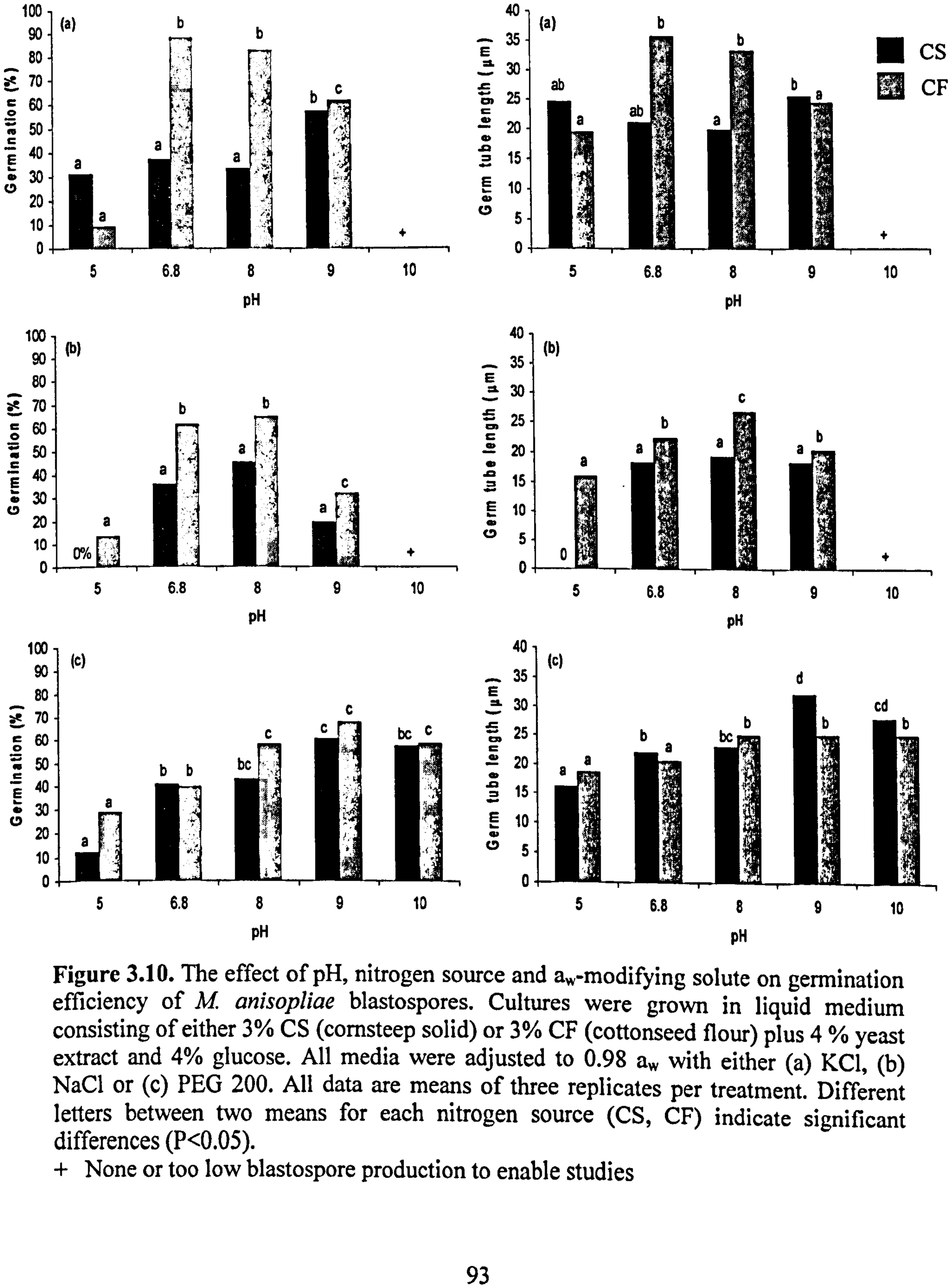


Figure 3.10. The effect of pH, nitrogen source and a_w-modifying solute on germination efficiency of *M. anisopliae* blastospores. Cultures were grown in liquid medium consisting of either 3% CS (cornsteep solid) or 3% CF (cottonseed flour) plus 4 % yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either (a) KCl, (b) NaCl or (c) PEG 200. All data are means of three replicates per treatment. Different letters between two means for each nitrogen source (CS, CF) indicate significant differences ($P<0.05$).

\+ None or too low blastospore production to enable studies

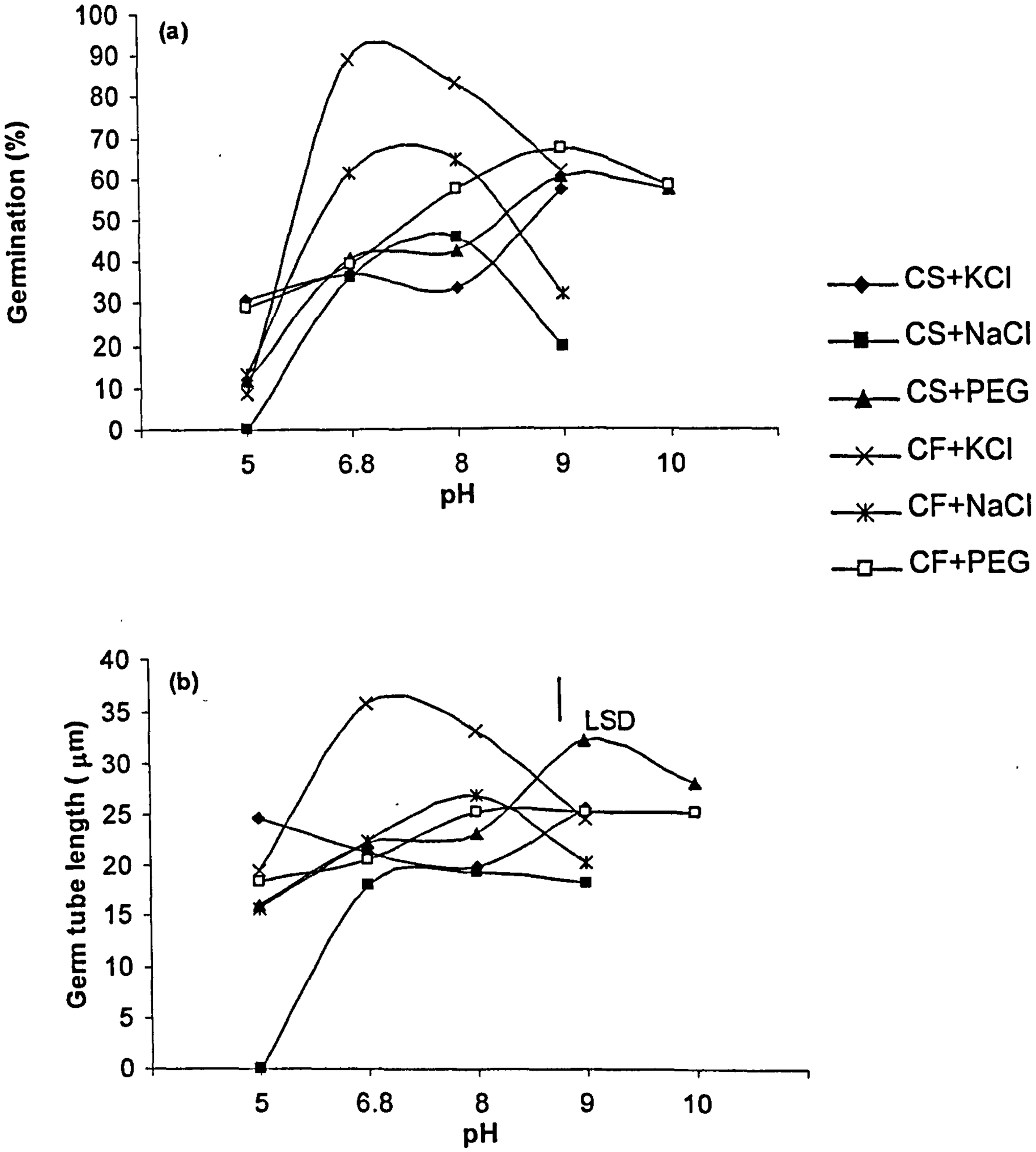


Figure 3.11. Representation of pooled results of the effect of pH, nitrogen source and a_w-modifying solute on germination efficiency (% germination and germ tube length) of *M. anisopliae* blastospores. Cultures were grown in liquid medium consisting of either 3% CS (cornsteep solid) or 3% CF (cottonseed flour) plus 4% yeast extract and 4% glucose. All media were adjusted to 0.98 a_w with either KCl, NaCl or PEG 200.

3.3.11. Selection of the best modified *M. anisopliae* blastospore treatments for biochemical and storage stability studies

Overall, optimum cultural conditions for combined high blastospore production, good quality characteristics (in terms of endogenous polyol and protein content) and high germination efficiency, occurred between pH 6.8 and 8 with the CF+YE as the nitrogen source and either KCl or NaCl as the modifying solute. Blastospores produced at pH 8 were preferred over the ones produced at 6.8 for subsequent biochemical and storage stability studies, because, although their volume was not evaluated, their bigger size could be translated into more endogenous reserves per spore. The treatments with the CS+YE as the nitrogen source and the same cultural conditions as the ones with the CF+YE nitrogen source were also included in these studies.

3.4 DISCUSSION

Impact of water-stress on blastospore production and ecological fitness

Blastospore production was greatly affected by the a_w of the medium and modified treatments reached a higher spore yield than the unmodified treatment. This was probably due to the effect of the a_w on the fungal morphology. It was evident in this study that unmodified cultures had a more viscous appearance than modified cultures after 48 h incubation and throughout the experimental period. However, cultures modified to 0.97 and 0.96 a_w using PEG 200 gave very low or no blastospore production. This could have been due to a potential toxicity of PEG 200 after a certain concentration or due to unfavourable rheological properties of the liquid medium caused by this compound. Similar observation has been reported by Frey & Magan (2001) with submerged cultures of *Ulocladium atrum*. Humphreys *et al.* (1989) found that increasing water-stress (from 0.986 to 0.980 a_w) resulted in a more filamentous morphology of the cultures exposing more of the biomass to the medium and therefore growth occured under more favourable conditions leading to a higher blastospore yield.

Impact of osmoprotection on endogenous reserves and ecological fitness

One aspect in the process of development of a fungal biocontrol agent, from production to formulation and storage, which has not been given much attention has been the

impact of harvesting procedure on the inoculum quality. In this study it has been shown that washing treatments after harvest had marked effects on endogenous polyol retention and subsequent germinability. It has already been mentioned that when fungi are grown under low environmental water potentials (water-stress conditions) they are able to lower their intracellular water potential accordingly by the production of polyols. Any subsequent exposure of such modified structures to an external environment with different water potential to the intracellular water potential, would subject these structures to an osmotic shock. In this study, blastospores produced under water-stress (0.98, 0.97 and 0.96 a_w), regardless of a_w-modifying solute, retained higher amounts of the low molecular weight polyol erythritol when they were washed with isotonic solutions equivalent to the growth medium, compared to washing with water. The difference in endogenous erythritol retention was particularly remarkable in blastospores produced under high water-stress (0.96 a_w). This suggested a rapid loss of high amounts of this polyol due to osmotic shock when such modified spores are washed with water. However, no difference in erythritol retention with either washing treatment was observed in blastospores from the 72-h-cultures modified to 0.97 a_w. This finding could be explained by the fact that blastospores from this treatment are young and that erythritol is synthesised *de novo* in the blastospores once they are released from the mycelium into the medium. This is further supported by the fact that very low amounts of erythritol (< 60 ppm) were detected in the water used to wash the blastospores from this treatment, while high amounts of erythritol were detected in the water used to wash the blastospores from the other water-stress treatments (see Table 3.4).

In previous work by Hallsworth & Magan (1995) polyol and trehalose content of wet-harvested *M. anisopliae* conidia was not significantly different from that of dry-harvested conidia, suggesting that polyols and trehalose did not leak out of characterised conidia when they were exposed to hypo-osmotic shock. The ease with which accumulated intracellular compounds leak out of a fungal structure depends on the nature of the structure and may also be species dependent. Luard (1982a) found that hypo-osmotic shock in *P. chrysogenum* and *C. fastidium* mycelium triggered a change in permeability to different polyols. Thus, the extent to which polyols are retained in the

fungal mycelium may be related to the xerophilic capabilities of a species (Hocking, 1986).

Higher endogenous retention of the high molecular weight polyol mannitol was also observed in blastospores washed with isotonic PEG 200 solution compared to blastospores washed with water. Contrary to the low molecular weight erythritol, no difference in mannitol retention with post-harvest washing treatment was found in blastospores produced under the highest water-stress (0.96 a_w). It is likely that mannitol was not synthesised in *M. anisopliae* blastospores produced under high water-stress, a result which is consistent with previous studies which suggested that with reducing a_w, fungi shift the balance of accumulation of compatible solutes away from the high molecular weight compounds such as mannitol and towards the low molecular weight compounds such as glycerol or erythritol (Kelly & Budd, 1991; Hallsworth & Magan, 1994a,b). Additionally, very low amounts of mannitol were detected in the amounts of water used for post-harvest washing of blastospores produced under high water-stress. This provides more evidence for very little mannitol synthesis under such conditions.

Intracellular accumulation of low molecular weight polyols also lowers intracellular water potential more effectively than higher molecular weight polyols. This reduction in intracellular water potential could confer an increase in germination rate of fungal propagules especially under low water availability conditions, since environmental water will move faster into a fungal spore (osmotic effect). In this study when germination was checked on a medium with freely available water, all treatments gave a high percentage germination (> 90%) suggesting that water was easily available to the spores under such conditions. However, when germination was checked on a medium with imposed water-stress (0.96 a_w) simulating drier conditions, there were remarkable differences in germination between treatments. Blastospores produced under unmodified conditions or modified to 0.98 a_w, germinated better when they had been washed with isotonic solutions compared to using water. An increased retention of erythritol by post-harvest washing with an isotonic solution could account for the increased germination in blastospores produced at 0.98 a_w but not for blastospores from the unmodified medium since no difference in erythritol retention by post-harvest

washing treatment was found for these blastospores. However, higher amounts of mannitol were retained in the unmodified blastospores with isotonic post-harvest washing treatment. Whether the increased amounts of mannitol could have accounted for the increased germination of unmodified spores washed with an isotonic solution is questionable since a saturated solution of mannitol has an a_w of 0.978 (Chirife *et al.*, 1984) and therefore would not enable depression of intracellular a_w sufficiently to allow improved osmoregulation on the water-stressed germination medium (0.96 a_w). It is possible that different compounds other than the ones investigated in this study could have been retained in the unmodified blastospores conferring better germination. For example, loss of ions due to alteration in membrane permeability caused by changes in turgor has been reported (Coster *et al.*, 1977; Luard, 1982c). However, a significant loss of intracellular compounds from unmodified blastospores would not be expected since such blastospores (intracellular water potential -2 MPa) are not exposed to a great osmotic shock when they are washed with water (water potential -0.1 MPa). Although PEG 200 is not utilised by fungi (Inch & Trinci, 1987; Humphreys *et al.*, 1989), a beneficial effect of PEG 200 at another step in the germination process should not be ruled out. The promotion of germination of blastospores washed post-harvest with isotonic solution should therefore be more closely investigated. The beneficial effect of post-harvest washing with isotonic solution, however was not observed with blastospores produced under 0.97 a_w and after 84 h of incubation and was detrimental for blastospores produced under 0.96 a_w level. It is likely that the higher amounts of PEG 200 needed to achieve an isotonic solution of 0.97 and 0.96 a_w are toxic to *M. anisopliae* and inhibit germination.

Regardless of a_w level, a_w-modifying solute and post-harvest washing treatment, blastospore germination considerably increased with culture age with the only exception being blastospores from the treatment modified to 0.96 a_w using KCl and which had been washed with an isotonic solution. The big increase in germination (from 0% to > 25%) with culture age for the water-washed blastospores from the 0.97 a_w treatments, where both erythritol and mannitol were at low levels, contrasts with their aforementioned role in germination improvement. It is likely that different mechanisms such as protein and RNA synthesis, breakdown of an inhibitor or release of a stimulant

intracellularly, or enzyme activity are also implicated during the germination process. The dependence of conidial germination on protein synthesis in different fungi has been demonstrated before, using either protein synthesis inhibitors or temperature sensitive mutants defective in this process (Cochrane & Cochrane, 1970; Loo, 1976; Osherov & May, 2000). Oliveira & Messias (1996) found that protein synthesis in *M. anisopliae* conidia increased with incubation time until germination. The complete blockage of germination of the newly-produced blastospores from the 72-h-cultures modified to 0.97 a_w, on water-stressed medium should not be attributed to absence of initiation of protein synthesis, as germination occurred at high levels (> 90%) on medium with freely available water. It is likely that the amounts of endogenous protein and/or of other endogenous reserves might have been very low in these blastospores affecting germination at low humidity levels. Endogenous lipids have been suggested to make a contribution in conidial germination under low relative humidity (Pupin *et al.*, 2000). Such studies were not performed in the present work. Clearly, fungal spore germination is a complex process but the germination promoting effect of post-harvest washing with an isotonic solution, when germination was checked on water-stressed medium, is clearly presented in this study for blastospores from cultures modified to 0.98 a_w where the isotonic PEG 200 solution used did not reach toxic levels.

The importance of physiological stage of blastospores was given particular attention when interpreting results. Any change in the physico-chemical properties of a medium results in a change of growth and the time of formation of reproductive structures of a fungal microorganism. Therefore, valid studies on the direct effect of such modifications on fungal inoculum quality, should take this into consideration. Previous studies have greatly neglected the effect of altering the physicochemical properties of a liquid medium on the time of spore production. For example Kleespies & Zimmermann (1998) tested the effect of different additives in liquid culture on the germinability and virulence of *M. anisopliae* blastospores by harvesting the spores from all treatments at a fixed incubation time (72 h), neglecting the effect of solutes on the a_w of the medium and on the time of spore production. In this way blastospores from different treatments could have been of different physiological stage at the time of harvest, making it difficult to interpret the outcome of such studies.

In the present study it was difficult to conclude whether the manipulation of endogenous reserves could have accounted for the increased germination of some treatments, but the beneficial effect of post-harvest washing with isotonic solution on retention of erythritol and mannitol was clear. Some studies have considered the effect of different types of washing on storage and freeze-drying (Cliquet & Jackson, 1999; Montazeri & Greaves, 2002) but they did not follow an accurate iso-osmotic washing of the harvested propagules. However, beneficial effects of different washing treatments were found in these studies.

Nitrogen screening

After optimum conditions (a_w: 0.98, a_w-modifying solute: PEG 200, culture age: 72 h) for high blastospore production of good quality were determined further enhancement in blastospore production was sought by screening different nitrogen sources provided at either 2, 4 or 7% at the above optimum conditions. Blastospore production increased with increasing nitrogen source concentration and for the best 3 nitrogen sources (CF, CS, BE) so did the final biomass, indicating that good mycelial growth is required to support high blastospore production. This is the first study of the effect of nitrogen source on blastospore production under steady-state a_w conditions. All studies that have considered the impact of nitrogen source on fungal spore production in liquid culture have neglected the effect that different sources of nitrogen or different amounts can have on the a_w of the medium. The effect of a_w on spore production in liquid culture is well documented (Inch & Trinci, 1987; Humphreys *et al.*, 1989; Frey & Magan, 2001).

Although good mycelial growth was required to support high blastospore production when CF, CS, or BE were used as the nitrogen source, increased mycelial growth did not always result in increased blastospore production. For example, MP supported very high mycelial growth but low blastospore production. It appears that some elements present in CF, CS and BE promote a higher blastospore production. It is very interesting to note that although CS and CF at all levels produced much higher blastospore numbers (up to 10-fold difference) compared to YE, in previous experiments it was shown that when CS and YE were used in combination (3% and 4% respectively) and always at 0.98 a_w, blastospore production surpassed that observed with either CS or CF

alone at all levels (2, 4 or 7%). The combined use of CS and YE (7% nitrogen source in total) did not produce higher biomass compared to CS alone (7%) suggesting that the enhanced blastospore production was not due to an increase in mycelial growth. It could be possible that the combined use of CS and YE results in a more filamentous growth of the fungus leading to improved oxygen transfer in the culture and subsequent increased blastospore production. The effect of increased filamentous fungal growth on improved blastospore production has been reported previously (Adàmek, 1963; Humphreys *et al.*, 1989; Kleespies & Zimmermann, 1998). Filamentous growth of *M. anisopliae* appears to be a prerequisite for blastospore induction as growth in pellets (when only inorganic nitrogen was provided) did not produce any blastospores.

Impact of nitrogen source, a_w-modifying solute and pH on M. anisopliae blastospore production and ecological fitness

Following the screening for the nitrogen source that supports high blastospore yield, the interaction between nitrogen source (CS + YE and CF + YE), pH and a_w-modifying solute on blastospore yield, quality and ecological fitness was evaluated. Regardless of nitrogen source and a_w-modifying solute optimum blastospore production occurred between pH 6.8 and 8 (except for CS+KCl) at which high final biomass was produced. In this pH range the CF was superior to CS in terms of blastospore production although not always producing higher final dry biomass, indicating once again that although good mycelial growth is required for good blastospore support, other factors are also implicated. According to previous studies on spore formation by fungi in liquid culture, it seems that blastospore formation greatly depends on biomass accumulation and morphology rather than nutrient depletion and subsequent spore induction. Several studies have suggested that some fungi like the fungal plant pathogen *C. truncatum* that must grow vegetatively and then differentiate to produce conidia, may require the depletion of essential nutrients to trigger sporulation (Jackson & Bothast, 1990; Schisler *et al.*, 1991), while fungi that produce 'yeast-like' blastospores like *M. anisopliae* benefit from rich media with optimal biomass accumulation (Jackson *et al.*, 1997; Vega *et al.*, 2003). Further studies that will investigate the morphology of *M. anisopliae* mycelial growth under the conditions studied can shed light on the interference and importance of fungal morphology on blastospore production. The present study has

identified the pH boundaries for *M. anisopliae* growth and sporulation in liquid culture. No growth occurred at pH 3 and sporulation was severely inhibited at pH 10. However, *M. anisopliae* can grow over a wider range of pH (2.9 to 11.1) on agar medium (Hallsworth & Magan, 1996). It is not known why growth is completely inhibited in liquid culture at pH 3 whereas it occurs on agar at the same value and should be further investigated.

As is shown in Table 3.6, the pH of the spent medium was lower than the initial pH at the end of the incubation time (72 h), apart from the case where the initial pH was adjusted to 3.5 and when it did not change. It seems that fungi regulate the pH of their external environment in order to create favourable conditions for their growth. This may be done by different processes including proton extrusion through the H^+-ATPase transport plasma membrane system, intracellular metabolism and plasma redox systems, which can be involved in the polyol metabolism (Jennings, 1995). Regulation of pH of the external environment should involve active processes and not passive diffusion of solutes, as when growth was inhibited (and most likely the metabolism as well) at pH 3.5, no change was observed in the medium pH during the incubation period.

Quality of blastospores in this multifactorial experiment was investigated in terms of the polyols, glycerol, erythritol, arabitol and mannitol and total protein content. Accumulation of increased amounts of endogenous low molecular weight polyols like glycerol and erythritol have been proposed to enhance spore germination under low environmental conditions by lowering the intracellular water potential and enabling faster water movement into the spore (Hallsworth & Magan, 1994b; Hallsworth & Magan, 1995; Pascual *et al.*, 2003). High concentrations of endogenous total protein may provide the amino acid pool necessary for protein synthesis and facilitate rapid germination. The dependence of conidial germination on protein synthesis in different fungi has been demonstrated before, using either protein synthesis inhibitors or temperature sensitive mutants defective in this process (Cochrane & Cochrane, 1970; Loo, 1976; Osherov & May, 2000). For these reasons it was decided to determine the conditions under which optimum amounts of these compounds occur in *M. anisopliae* blastospores. Glycerol was not detected in any of the treatments and arabitol was

present in very low amounts (< 2.5 mg g^{-1} f.w.). The prevailing polyols for all conditions tested were the lower molecular weight erythritol and the high molecular weight mannitol. This shows that in *M. anisopliae* blastospores the prevailing low molecular weight polyol is erythritol whereas in conidia produced under solid state fermentation (see Chapter 2) the prevailing low molecular weight polyol is arabitol. Highest concentrations of both the low molecular weight polyol erythritol and of the high molecular weight polyol mannitol were detected between pH 6.8 and 8 regardless of nitrogen source and a_w-modifying solute. The accumulation of erythitol with increasing pH up to 8 is consistent with studies with *Aspergillus nidulans* in liquid culture where under two different nitrogen status regimes (ammonium and nitrate), the polyol appeared or increased respectively with increasing pH from 3.5 to 6.5 (Dijkema *et al.*, 1986), but contrasts with studies with *M. anisopliae* on agar where endogenous erythritol and mannitol concentration in conidia did not change over pH range 3 to 10 (Hallsworth & Magan, 1996). Whether the difference between liquid culture and semi-solid culture in response to pH changes in terms of polyol accumulation is due to differences in strains or in the nature of the culture needs to be investigated.

It is interesting to note that there was a remarkable difference in polyol (erythritol and mannitol) concentration over the pH range from 5 to 10 when KCl and NaCl were added to the medium to modify the a_w. When the non-ionic solute PEG 200 was used as the a_w-modifying solute the polyol concentration did not change considerably over the same pH range. Additionally, much higher amounts of erythritol and mannitol were accumulated endogenously in blastospores when KCl and NaCl were employed for a_w modification compared to PEG 200. Slayman & Slayman (1970) examined the kinetics of net potassium uptake as a function of potassium concentration and pH of the medium, and they found that at low pH (4.0-6.0) the net flux is a simple exponential function of time that obeys Michaelis-Menten kinetics as a function of potassium concentration, whereas at high pH, potassium uptake is more complex, obeying sigmoid kinetics. It has been proposed that sodium uptake occurs via the potassium transport systems so that could be well affected by changes in pH. This differential uptake of potassium and possibly of sodium in relation to pH may account for the remarkable

differences in polyol concentration over the pH range tested when KCl and NaCl were the a_w-modifying solutes.

When CF+YE was used as the nitrogen source, erythritol and mannitol concentration was higher than when CS+YE was used. Although the effect of nitrogen source on polyol accumulation in fungi has been studied before (Dijkema *et al.*, 1986) this is the first report for *M. anisopliae*. This is also the first report on the effect of pH and nitrogen source on the endogenous accumulation of total protein in *M. anisopliae*. As in the case of polyols, maximum protein concentration occurred between pH 6.8 and 8 with CF resulting in higher amounts of endogenous protein compared to CS. It should be noted however that in order to attribute a direct effect of pH, and/or of the solutes used for a_w modification on the differences in polyol and total protein concentration, blastospores should be of the same physiological age. Different liquid environmental conditions affect the kinetics of fungal growth and therefore at a certain incubation time different liquid treatments can be of different physiological age.

The length of blastospores increased with increasing pH of the liquid medium but the width remained constant. This shows that the reported increased amounts of endogenous polyols and protein per g of fresh blastospores produced between 6.8 and 8 values of pH translates into increased amounts of these compounds per blastospore.

It is very interesting to note that enhanced germination (expressed as % germination and germ tube length) under water-stress conditions occurred with blastopores containing optimum amounts of endogenous polyols and protein (blastospores produced when CF+YE was the nitrogen source, KCl or NaCl was employed for a_w modification, and the pH was adjusted between 6.8 and 8). It was very good to observe that optimum blastospore production also occurred under the same conditions. In this study a comprehensive study for the production of high number of *M. anisopliae* blastospores with increased ecological fitness and at very low costs is presented.

Chapter 4 ENZYME AND TOXIN PRODUCTION

4.1 INTRODUCTION

The fungal entomopathogen *M. anisopliae* penetrates the proteinaceous cuticular barrier of insects through a combination of mechanical pressure and enzymic degradation (St. Leger *et al.*, 1986a). This fungus produces extracellular enzymes with activity against the major components of insect cuticle, namely protein, chitin and lipid (St. Leger *et al.*, 1986b). Although to date there is no solid evidence that the production of such enzymes are determinants of pathogenicity of fungal entomopathogens, several studies have correlated production of proteases and chitinases with successful and/or enhanced virulence (Coudron *et al.*, 1984; Jackson *et al.*, 1985; St. Leger *et al.*, 1988a; St. Leger *et al.*, 1992). The ability of a fungal entomopathogen to produce degradative enzymes *per se* should not be considered as an indication of pathogenicity since many fungal species not associated with disease also produce such enzymes. However, these enzymes could contribute significantly to disease development in insects.

Studies with *M. anisopliae* have suggested that proteases might be very important in facilitating fungal invasion in the host and subsequent disease development. Chitin fibrils are masked with protein and the rapid and high production of proteolytic activity (< 24 h) on insect cuticle, would be an advantage in pathogenesis, providing nutrients and enabling the onset of cuticle penetration before host defences become effective (St. Leger *et al.*, 1986c). Additionally, strains of *M. anisopliae* produce a high complexity of protease isoforms with different inducible determinants; growth on different substrates (elastin, cellulose, protein bovine serum albumin) induces different isoforms (St. Leger *et al.*, 1994). This provides the organism with the ability to degrade the variable cuticle substrates that they encounter in the diverse host insects that they infect. Chitinase on the other hand, is considered to have a secondary role in host invasion by *M. anisopliae* as it is produced later than proteases and is tightly regulated by chitin degradation products (St. Leger *et al.*, 1986c, d). Therefore, chitinase is required for only a brief period during penetration of host cuticle.

As far as the operative steps in enzyme regulation and secretion by *M. anisopliae* is concerned, the two major proteases Pr1 and Pr2 currently provide the best understood model. Studies with metabolic inhibitors indicated synthesis is substantially reduced by inhibitors of transcription and translation, suggesting that the operative steps in protease regulation involve *de novo* synthesis of mRNA and protein (St. Leger *et al.*, 1988b; St. Leger *et al.*, 1991). However, inhibition of DNA synthesis did not inhibit Pr1 and Pr2 production. The secretion of active mature protease has been shown to involve synthesis of high molecular mass precursors which are processed down to the low molecular mass mature extracellular enzyme (St. Leger *et al.*, 1991). Secretion of proteases via the cell membrane is an active process as inhibition of the plasmalemma ATPase inhibits release of the enzyme.

Once the cuticle has been breached, the fungus is believed to kill the host by some combination of mechanical damage produced by fungal growth, nutrient exhaustion and toxicosis. The relative importance of these mechanisms varies with the specific fungal isolate or host. *M. anisopliae* has been found to produce a family of cyclic peptide toxins, called destruxins. The first report of the discovery of these toxins comes from Kodaira in 1961. Since then a large number of studies of this family of compounds has been published. Although the insecticidal activity of these compounds is well documented (see Pedras *et al.*, 2002, for a review), there is not always a direct link between high-producing strains of destruxin and increased insect mortality, since fungal strains with high production of destruxins can have the same LT_{50} (4 days) with low destruxin producing strains (Amiri-Besheli *et al.*, 2000). It should be noted that it is rather difficult to correlate data from both *in vivo* and *in vitro* production of destruxins with insecticidal activity, firstly because destruxin production might be different in the insect haemolymph than in a culture medium and secondly due to potential biotransformation of destruxins in the haemolymph. However, there is strong evidence in the literature that destruxins are important determinants of virulence.

Destruxins are cyclic depsipeptides consisting of five basal amino acids and an α-hydroxy acid, which constitute the basal structure of destruxins. Therefore, the availability of amino acids could be a pivotal factor in biosynthesis of destruxins. Liu *et*

al. (2000) have shown that addition of different amino acids in the culture medium highly influenced destruxin production. Products of secondary metabolites will be influenced by the endogenous compounds of a cell that produces them.

In this study, the question of whether the differences in endogenous total protein content of modified blastospores and conidia could be translated into different enzyme and destruxin A production was investigated. Endogenous protein content may serve as an amino acid pool necessary for *de novo* synthesis of protein and possible enzyme and/or destruxin production. The effect of post-harvest osmoprotection of modified blastospores by different isotonic solutions on enzyme secretion was also investigated. Osmoprotection results in retention of higher amounts of endogenous polyols and potentially of different other elements and/or compounds which might be involved in enzyme regulation. Results were compared with the unmodified blastospore treatment and the best conidial treatment from the solid substrate experiment.

4.2 MATERIALS AND METHODS

4.2.1 Fungal species

As described in Section 2.2.1

4.2.2 Enzyme studies

Inoculum preparation

The selected 4 best *M. anisopliae* blastospore treatments and the best conidial treatment were tested for extracellular protease and chitinase activity. These treatments were selected based on spore yield and quality characteristics in terms of endogenous reserves and germination efficiency after optimisation as described in the solid and liquid fermentation chapters (chapters 2 and 3). The cultural conditions and the post-harvest washing treatments are summarised in Table 4.1. Details of media preparation, culture conditions and harvest of conidia and blastospores were described in sections 2.2.5 and 3.2.6 respectively. In the case of modified blastospore treatments, the effect of post-harvest hypo-osmotic shock versus post-harvest maintenance of iso-osmotic conditions to the growth medium on extracellular enzyme activity was also examined.

Post-harvest hypo-osmotic shock was accomplished by re-suspending of harvested blastospores in sterile 10 ml de-ionised water after harvest as described in section 3.2.3. Post-harvest iso-osmotic conditions to the growth medium (0.98 a_w) were achieved by re-suspending harvested blastospores in sterile 10 ml solutions of either glucose (18.73%), NaCl (3.55%) or PEG 200 (15%). The conidial treatment was harvested by flooding the cultures with sterile de-ionised water as described in section 2.2.5. After re-suspension all samples were centrifuged at 2700 r.p.m. for 10 min and subsequently the supernatant discarded. The resultant blastospore and conidial pellets were used for spore suspension preparations that served as the inoculum for enzyme studies. Blastospores produced in the unmodified liquid medium as described in section 3.2.2 were used as the control treatment.

Table 4.1. Culture conditions and post-harvest treatment of *M. anisopliae* characterised propagules used for extracellular enzyme studies.

Substrate	**Culture age**	**pH**	**a_w**	**a_w-modifying solute**	**Washing solution**
Solid;bulgar wheat	5d	ND	0.98	none	Water
				KCl	Water
					PEG 200
					NaCl
Liquid;CS+YE+Gl	72h	8	0.98		Glucose
					Water
				NaCl	PEG 200
					NaCl
					Glucose
					Water
				KCl	PEG 200
					NaCl
Liquid;CF+YE+Gl	72h	8	0.98		Glucose
					Water
				NaCl	PEG 200
					NaCl
					Glucose

CS: cornsteep solid (3%), CF: cottonseed flour (3%), YE: yeast extract (4%), Gl:glucose (4%)
ND: not determined

Enzyme induction

The medium used for enzyme induction consisted of a basal salts medium (0.06% $MgSO_4$, 0.05% NaCl, 1.5% KH_2PO_4, 0.01% $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4$) supplemented with 1% chitin from Crab Shells (Sigma, U.K.). The pH of the medium was adjusted before autoclaving to 6.3 using 40% NaOH. This medium was selected after an optimisation procedure that involved different substrates (chitin;1%, cellulose;1%, gelatine;1%, chitin+gelatine;0.5+0.5%, or chitin+cellulose;0.5+0.5%) and pH values (5, 6.3, 7 and 8) that have been reported previously in the literature (St. Leger *et al.*, 1988b; St. Leger *et al.*, 1986c; St. Leger *et al.*, 1998), to determine optimum conditions for high chitinase and protease activities. Each treatment (as described in Table 4.1) was carried out in triplicate 250 ml Erlenmeyer flasks each containing 50 ml of enzyme medium. After autoclaving at 120°C for 15 mins, each flask received 0.5 ml of a treatment inoculum of 3.5×10^7 spores ml^{-1}, made in the same sterile solution as the one used for post-harvest washing of the spores of that treatment. Enzyme cultures were incubated at 25°C on a rotary shaker at 180 r.p.m.

Preparation of culture filtrate for enzyme assays

Subsamples (2 ml) were taken after 24, 48, 72, and 96 h incubation and divided in two equal parts (1 ml) in two Eppendorf tubes (1.5 ml). These were centrifuged at 13000 r.p.m. for 10 mins and the supernatants were filtered through 0.2 μm hydrophilic filters into clean Eppendorf tubes. One tube from each replicate was boiled in a water bath for 15 mins and served as the control for each treatment.

Enzyme assays

(a) Chitinase activity

Carboxymethyl-substituted (CM-) chitin, labelled covalently with Remazol Brilliant Violet 5R (RBV) was used as a substrate for the assay of chitinase activity (Wirth & Wolf, 1990). The enzyme substrate was purchased from Roewe, Germany. Enzyme assays were performed in 96 well microplates. Dye-labelled substrate (CM-chitin-RBV) in aqueous solution (2 mg ml^{-1}) and buffer (0.2 M sodium acetate-acetic acid buffer, pH 5) were equilibrated at 37°C for 1 h. After equilibration, 50 μl of CM-chitin-RBV followed by 50 μl of sodium acetate-acetic acid buffer were placed in each well in a

microtitre plate. Subsequently, 100 µl of culture filtrate from a treatment were added in a well. For each treatment, control wells were prepared by adding 100 µl of boiled culture filtrate of the corresponding treatment. The plates were sealed with a low evaporation lid and incubated for 30 mins at 37°C. The reaction was terminated by the addition of 50 µl of HCl (1 N) in each well, causing precipitation of the non-degraded high-polymeric substrate. Subsequently, the plates were cooled on ice (10 min) and centrifuged at 3000 r.p.m for 10 mins. Supernatants (150 µl), containing soluble, dye-labelled degradation products were transferred to a 96-well, half-size EIA plate (175 µl cavities, Costar, 1 cm cuvette length) and measured spectrophotometrically at 530 nm against controls prepared similarly by adding 100 µl of boiled culture filtrate of the corresponding treatment.

The chitinase assay incubation time (30 mins) was determined after an optimisation step, by testing different incubation times (30 mins, 1 h, 2 h, 3 h) and concentrations of a commercial chitinase from *Streptomyces griseus* (Sigma, UK) (from 0.05 units ml^{-1} to 0.0025 units ml^{-1}). The rest of the assay conditions had been optimised previously (Wirth & Wolf, 1992). One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – boiled sample absorbance) x 1000 x min^{-1}.

(b) Protease activity

The protease activity in the filtrate was quantified using sulphanilamide azocasein substrate (Germano *et al.*, 2003), purchased from Sigma (A-2765), and the assay optimised in 96 well microplates instead of cuvettes. Azocasein is a chemically modified protein, prepared by adding sulphanamide groups to casein, which are orange and covalently linked to the peptide bonds. When azocasein is subject to proteolytic action short peptides and amino acids are liberated from the chain and remain in solution, giving an orange colour to the solution. The greater the proteolytic activity the more intense the orange colour of the solution. Following incubation the assay was carried out at 45°C using 30 µl of azocasein (1% in 0.2 M Tris-HCl buffer, pH 7.5) and the reaction started by adding 20 µl of culture filtrate. After incubation for 1 hour the enzyme was inactivated by the addition of 150 µl of trichloroacetic solution (10% w/v) and this solution was neutralised using 50 µl of NaOH 1M. Trichloroacetic acid (TCA)

was added to stop the reaction and to precipitate macromolecules, including the enzymes and the undigested azocasein. These were then removed by centrifuging the microplate at 3000 r.p.m., for 10 mins, in a centrifuge equipped with a rotor for microplates. Subsequently, supernatants (150 µl) were transferred to a 96-well, half-size EIA plate (175 µl cavities, Costar, 1 cm cuvette length). The absorbance was measured spectrophotometrically at 440 nm against a blank prepared similarly by adding 100 µl of boiled culture filtrate of the corresponding treatment. One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – boiled sample absorbance) x 1000 x min^{-1}. This assay was first optimised using commercial protease from *Aspergillus oryzae*, 500 units g^{-1} (Sigma, UK), giving a positive result for concentrations as low as 0.0005 units of protease in the well.

4.2.3 Destruxin A studies

Inoculum preparation

As described for enzyme studies

Media peparation

Czapek Dox broth (Sigma, UK) supplemented with 0.5% bacteriological peptone (LAB M) was used as the medium for destruxin A studies, because it has been widely used previously (Loutelier *et al.*, 1996; Amiri *et al.*, 1999; Amiri-Besheli *et al.*, 2000; Hsiao & Ko, 2001; Wang *et al.*, 2003). Each treatment (as described in Table 4.1) was carried out in triplicate 250 ml Erlenmeyer flasks each containing 100 ml of liquid medium. After autoclaving at 120°C for 15 mins, each flask received 0.5 ml of a treatment inoculum of 3.5 x 10^7 spores ml^{-1}, made in the same sterile solution as the one used for post-harvest washing of the spores of that treatment. Liquid cultures were incubated at 25°C on a rotary shaker at 180 r.p.m.

Preparation of culture filtrate for destruxin A determination

Subsamples (2 ml) were taken after 3, 4, 5, and 7 days incubation and placed in 2 ml Eppendorf tubes. These were centrifuged down at 13000 r.p.m. for 10 mins and the supernatants were filtered through 0.2 µm hydrophilic filters into clean Eppendorf tubes. Samples were analysed after filtration without any extraction with chemicals as it

has been reported that chromatograms from crude culture filtrate samples are very similar to those obtained from extracts with CH_2Cl_2-AcOEt (50:50, v/v) (Loutelier *et al.*, 1996). Cultures after 7 days of incubation were too viscous to allow any sampling.

Chromatographic system

Samples were analysed using a Gilson HPLC (715) system equipped with a Gilson UV detector (117). A Phenomenex column (250 x 4.60 mm i.d.) packed with Lichrosorb 100 RP – 18 (5μm) was used at a flow rate of 1.0 ml min^{-1}. The mobile phase was a linear gradient of acetonitrile:HPLC grade water 30:70 to 50:50 in 25 min and 100% acetonitrile for a further 5 min with monitoring at 215 nm. The mobile phase was sonicated for 10 min, and de-gassed with helium before it was used. Fifty μl of sample were injected. Five standard solutions made up in acetonitrile:HPLC grade water 30:70 of between 2 and 50 ppm were injected onto the column immediately before samples in order to calibrate the system. Standard solutions were prepared using a commercial destruxin A purchased by Sigma, U.K.

4.2.4 Statistical analysis

As described in Section 2.2.9

4.3 RESULTS

4.3.1 Chitinase activity

All blastospore treatments (control and modified) regardless of post-harvest washing treatment produced none (up to 48 h incubation) to very low amounts of extracellular chitinase activity (0.1-0.3 units; after 96 h incubation) (data not shown). However, conidia from the bulgar wheat treatment produced chitinase activity after 48 h of incubation which reached up to 1 unit after 96 h incubation (data not shown).

4.3.2 Protease activity

In contrast, blastospore treatments (modified and control), regardless of post-harvest washing treatment, showed extracellular protease activity after 24h incubation, compared to bulgar wheat conidia, which only showed extracellular protease activity after 48 h incubation (Figure 4.1). Additionally, at the end of the incubation period (96

h) all blastospore treatments reached a higher protease activity compared to conidia. For all modified blastospore treatments, post-harvest washing with isotonic PEG 200 solution resulted in increased protease activity throughout the incubation period, compared to all the other post-harvest isotonic washing treatments and to unmodified blastospores and the conidial treatment. This enhanced protease activity by post-harvest washing with isotonic PEG 200 was significantly different ($P<0.05$) when compared to protease activity by modified blastospores washed with water, unmodified blastospores and the conidial treatment after 72 h incubation. Osmoprotection with PEG 200 solution of modified blastospores produced in the medium with CF+YE nitrogen source (regardless of a_w-modifying solute) resulted in the highest enzyme activity after 96 h incubation compared to all other treatments.

4.3.3 Destruxin A

Production of extracellular destruxin A varied with post-harvest washing treatment, and the medium cultural conditions for blastsopore production (Figure 4.2). However, regardless of post-harvest washing treatment, all modified blastospore treatments produced higher amounts of extracellular destruxin A after 5 days incubation compared to unmodified blastospores (except for the CS+NaCl treatment washed with the isotonic NaCl treatment). Destruxin A production by the conidial treatment was also enhanced compared to the unmodified blastospore treatment after 5 days incubation.

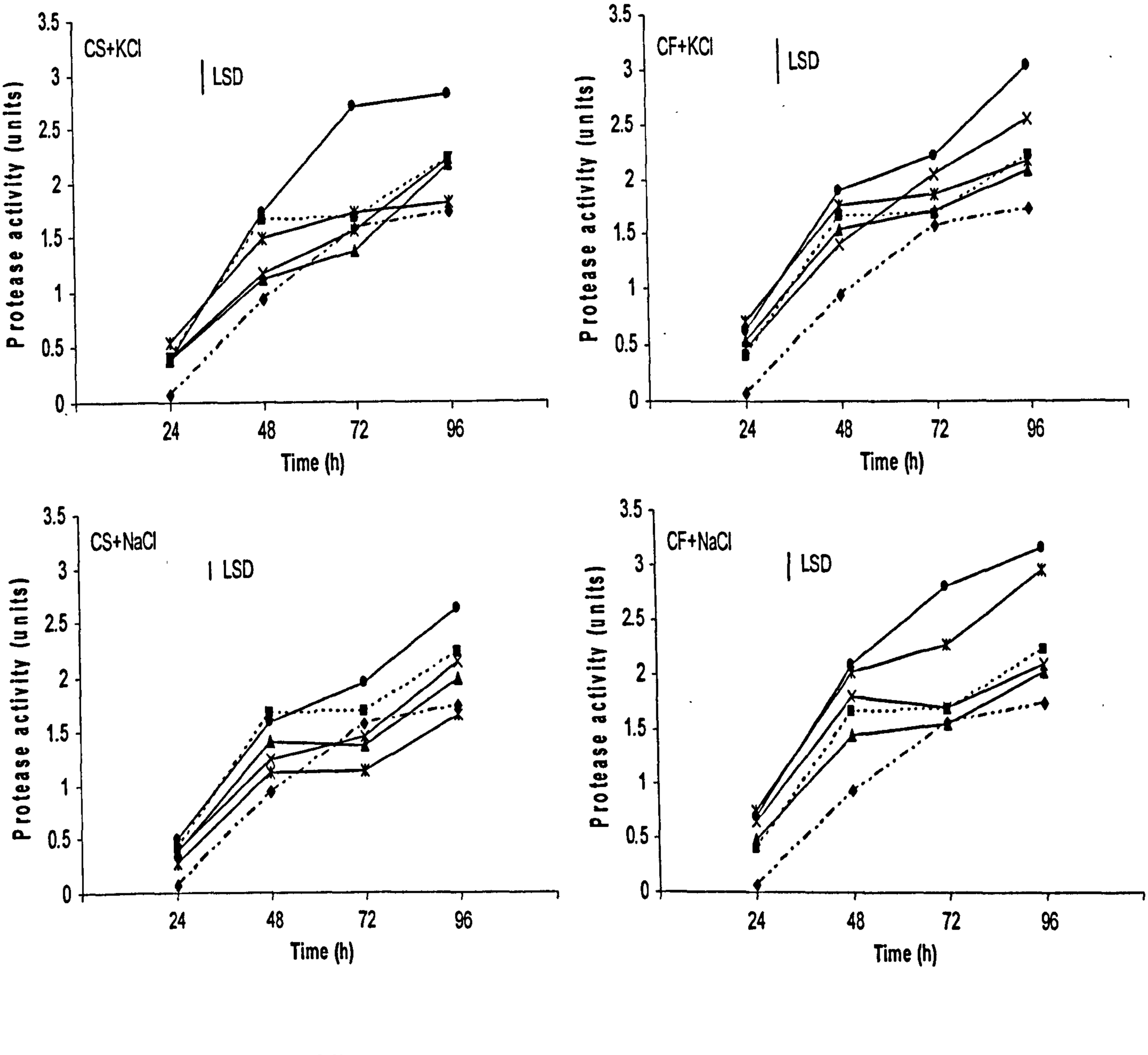


Figure 4.1. Effect of different post-harvest washing treatments on extracellular protease activity of modified *M. anisopliae* blastospores in relation to unmodified blastospores (control) and solid-substrate produced conidia. Culture conditions and post-harvest treatment of modified *M. anisopliae* blastospores and conidia are described in Table 4.1.

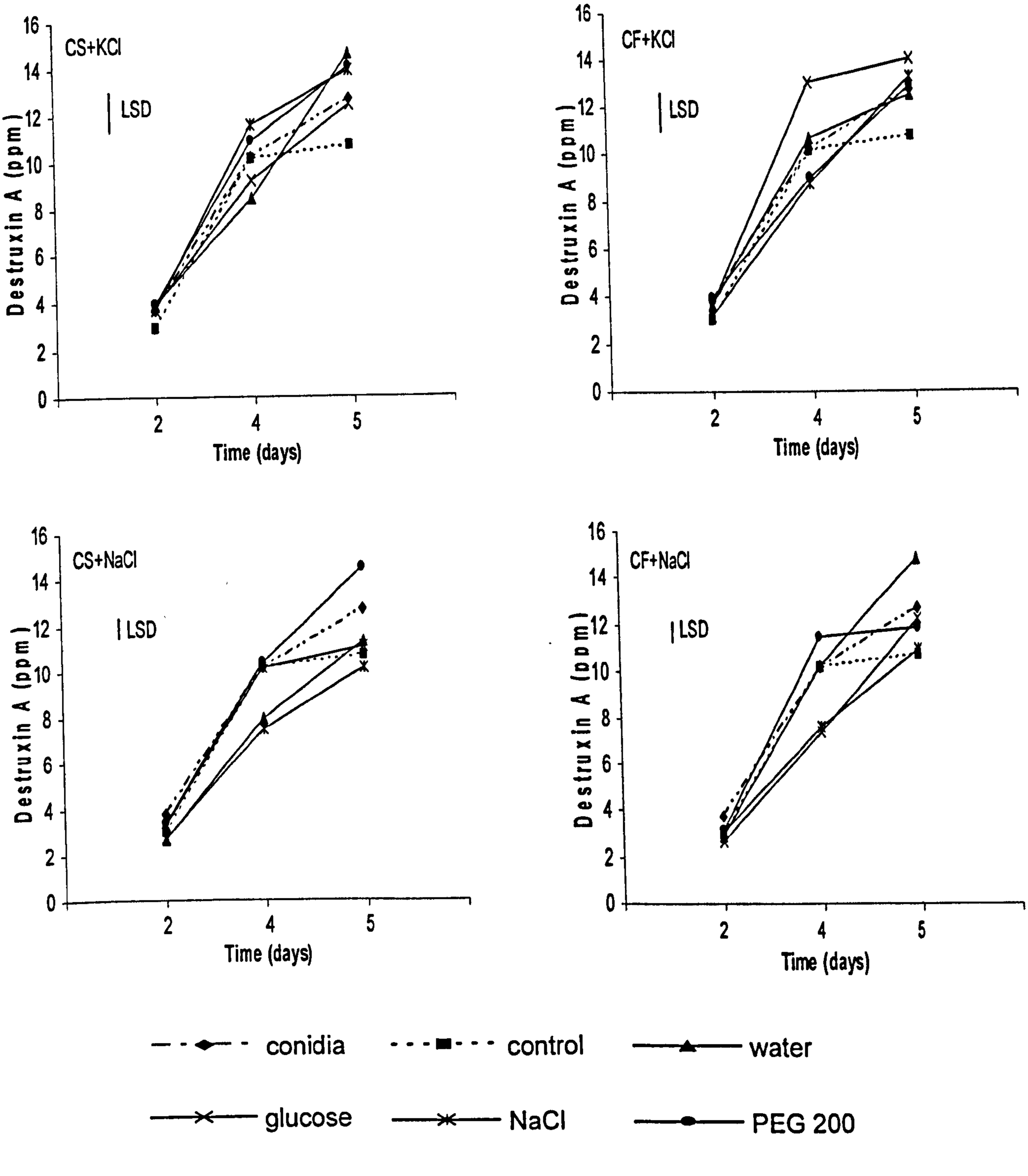


Figure 4.2. Effect of different post-harvest washing treatments on extracellular destruxin A production by modified *M. anisopliae* blastospores in relation to unmodified blastospores (control) and solid-substrate produced conidia. Culture conditions and post-harvest treatment of modified *M.anisopliae* blastospores and conidia are described in Table 4.1.

4.4 DISCUSSION

The aim of this study was to evaluate whether the different profiles in endogenous protein of different modified *M. anisopliae* blastospore treatments, and the retention of higher amounts of polyols by osmoprotection of modified blastospore treatments, had any effect on the production of the extracellular enzymes protease and chitinase and extracellular destruxin A. In order to eliminate as much as possible the effect of different germination rates of different spore treatments on enzyme activity, the study was carried out in liquid medium with no imposed water-stress, since it was found that under conditions with fully available water, all blastospore treatments gave equally very high germination (too extensive to allow any measurements) after 12 h incubation on water agar (data not shown). However, the conidial treatment had a slower germination under such conditions (see Table 2.2). The results showed that osmoprotection of all modified blastospore treatments with isotonic PEG 200 solution resulted in increased extracellular protease activity compared to blastospores subjected to hypo-osmotic shock (post-harvest washing with water) and to unmodified blastospores. The other isotonic washing treatments however did not result in a consistently higher protease activity for all modified blastospore treatments. It is probable that glucose and NaCl interfered with protease activity. In support of this St. Leger *et al.* (1988b), showed that production of proteases is repressed by addition of glucose in a medium containing protease inducible substrate through catabolite repression, and that protease activity is inhibited by the presence of KCl through interference with adsorption onto the cuticle (1986b). Since PEG 200 is not utilised by fungi, it is likely that it does not interfere with enzyme activity and that retention of higher polyol amounts through osmoprotection with this solution results in increased enzyme activity. Endogenous protein content of osmoprotected modified blastospores should not have affected extracellular protease production, since osmoprotection by isotonic PEG 200 solution and hypo-osmotic shock of a modified blastospore treatment (CF+KCl, pH 8, a_w 0.98) did not show any difference in protein content (data not shown). The fact that all blastospore treatments produced higher levels of protease activity after 96 h of incubation compared to the conidial treatment is more likely due to increased germination rates of blastospores. More detailed studies that determine growth rates of different treatments at all sampling

time are required to determine whether osmoprotection of modified propagules can lead to enhanced enzyme activity.

It is very interesting to note that although germination of the conidial treatment is slower than any of the blastospore treatments, chitinase activity by this treatment at the end of the incuation period (96 h) was higher (1 unit) than the very low activity observed for blastospores (< 0.3 units). This shows some enhanced production of extracellular chitinase by this conidial treatment in comparison with blastospores.

The results of the effect of osmoprotection of modified blastospores on destruxin A production varied with the medium used for blastospore production and the type of isotonic washing solution. The only enhanced production of extracellular destruxin A was observed with the CS+NaCl and the CF+KCl treatments washed with isotonic PEG 200 and glucose solutions respectively. Again in this case more detailed studies that determine the growth rates of different treatments are required in order to reach more accurate conclusions about the effect of osmoprotection on extracellular destruxin A production. However, the finding that none of the osmoprotectant solution substantially inhibited production of destruxin A is a desirable outcome since the beneficial effects of osmoprotection (retention of higher endogenous amounts of polyols, enhanced germination) can be retained without any interference with the production of this metabolite that could contribute to improved pathogenicity.

Chapter 5 STORAGE STABILITY

5.1 INTRODUCTION

To have a practical use, fungal biological control agents should possess adequate shelf-life. The major obstacle in the commercialisation of biocontrol products is the development of a formulated product with long stability in storage. A fungal BCA must retain its stability for at least one year to be considered for commercial development (Rodham *et al.*, 1999). Stability for 18-24 months at room temperature (25°C) is required to increase market competitiveness, but it has remained an elusive goal (Wraight *et al.*, 2001). There are several ways by which the stability of a product can be improved and include a) selection of fungal species or strain, b) appropriate production conditions, c) post-production processing and d) addition of certain additives in the formulation product. The ways by which such procedures can result in improved storage stability of a fungal BCA have already been described in Section 1.8.

Most studies of formulation of BCAs have considered the steps during production and drying of the desired product. One step that has not been given sufficient attention is the handling of the final fungal product during harvest. Methods of production of fungal BCAs aim at the production of propagules with modified levels of endogenous reserves, like polyols, sugars, lipids, which could be beneficial for subsequent stability and efficacy of the product. However, any inappropriate handling of the produced cells during harvest can result in loss of considerable amounts of desirable endogenous reserves. Many studies have used immersion in water as a method of harvesting the produced fungal propagules (Jin *et al.*, 1991; Fargues *et al.*, 1994; Costa *et al.*, 2000; Fargues *et al.*, 2001), a procedure which may result in leakage of intracellular compounds out of the cell. When cells are grown in modified medium (especially liquid medium) they adjust their intracellular water potential according to the water potential of the external environment (Luard, 1982a). Subsequent immersion in water results in hypo-osmotic shock and possible loss of endogenous compounds. Such an effect has been reported previously for the obligate xerophilic fungus *C. fastidium* (Luard, 1982b).

It is now widely accepted that different sugars protect fungal membranes during dehydration, and several studies have tested the effect of immersion of fungal propagules in sugar solutions before a drying process (Cliquet & Jackson, 1999; Montazeri & Greaves, 2002), with successful outcomes. However, there has been no study of the effect of an accurate harvest washing of fungal propagules using iso-osmotic solutions to the growth medium on drying resistance and subsequent storage stability. The objective of this study was to determine whether maintenance of iso-osmotic conditions during post-harvest washing of *M. anisopliae* characterised blastospores with modified endogenous levels of polyols could lead to increased drying resistance and storage stability. Fluidised-bed drying and freeze-drying were examined to test the drying resistance. Storage stability was evaluated as wet pastes and freeze-dried spores.

5.2 MATERIALS AND METHODS

5.2.1 Fungal species

As described in Section 2.2.1

5.2.2 Wet pastes

Spore production and storage

The 4 best *M. anisopliae* blastospore treatments (shown in Table 4.1) were tested for their storage stability as wet pastes. Blastospores produced in unmodified Adámek's medium (1963) prepared as described in section 3.2.2, were also included in the study. Details of media preparation, culture conditions and harvest of the best blastospore treatments are described in section 3.2.6. As with enzyme studies, the effect of post-harvest hypo-osmotic shock versus post-harvest maintenance of iso-osmotic to the growth medium conditions was also examined. This was achieved by re-suspension of harvested blastospores in sterile 10 ml de-ionised water after harvest or by re-suspending blastospores in sterile 10 ml solutions of either glucose (18.73%), NaCl (3.55%) or PEG 200 (15%) which were iso-osmotic to the growth medium (0.98 a_w), as described previously (see Section 4.2.2). After re-suspension, 1 ml of a spore suspension was placed in sterile Eppendorf tubes (1.5 ml). These were centrifuged at

3000 r.p.m. for 10 mins and after that the supernatant was discarded. Eppendorf tubes were divided in two batches and one set was stored at 4°C and the other at 25°C. All treatments were done in triplicate.

Germination studies

Storage stability of the selected *M. anisopliae* spore treatments was checked by testing germinability on agar medium with fully available water (0.998 a_w) and with imposed water-stress (0.963 a_w). Germination media were prepared as described in section 2.2.6. Germination studies were done at time 0 and after 2, 6 and 12 weeks storage. Germination media were inoculated with spores from a treatment by spreading 100 µl of spore suspension, made up in 10 ml of the same sterile isotonic solution or water used to wash the spores after harvest in the case of the modified best treatments and in 10 ml of sterile distilled water in the case of the unmodified treatment. Petri plates of the same a_w were sealed in a polyethylene bag and incubated at 25°C. Samples were taken after 12 h incubation in the case of media with fully available water and after 84 h on media with imposed water-stress. These sampling times were optimised after preliminary experiments. Percentage germination and germ tube length were assessed as described in Section 2.2.6.

5.2.3 Fluidised bed drying

Spore production and drying

(a) Screening for optimum drying temperature and time

Initially, different temperatures (30, 40, 50 and 60°C) and duration (10 and 20 mins) of drying were evaluated. Blastospores used for this screening procedure were produced in the liquid treatment modified to 0.98 a_w using KCl and pH 8. This treatment was selected as it supported good blastospore yield, providing enough material to work with. Blastospores were harvested as described in Section 3.2.3. After harvest and removal of the spent medium blastospores were washed in 10 ml sterile distilled water, centrifuged at 2700 r.p.m. for 10 min and the supernatant removed. The pellet from 3 flasks was placed on a layer of Whatman filter paper (42, 12.5 cm) and left for 5 mins to remove any excess of water. After that the amount of pellet was placed in a 2 ml plastic syringe and that was syringed out in a tube in the fluidised bed-dryer. After drying, viability of

blastospores was checked by staining the spores with viablue and examining them under fluorescent microscopy. Fluorescence was restricted to a bright outline (halo) in the case of live spores, whereas dead cells fluoresced brightly throughout. This rapid method of estimation of viability has been described for yeast cells by Hutcheson *et al.* (1988) and was evaluated for *M. anisopliae* blastospores and conidia showing excellent validity for this type of cells. The validation of this method for *M. anisopliae* blastospores and conidia is shown in Appendix II. All treatments were done in triplicate.

An amount of dried spores from each treatment was used for moisture content determination. For this reason dried spores were placed in 1.5 ml Eppendorf tubes and left overnight in an oven set at 70°C.

(b) Effect of osmoprotection on drying tolerance

The effect of post-harvest hypo-osmotic shock versus post-harvest maintenance of iso-osmotic to the growth medium conditions was also examined for fluidised bed drying tolerance. This was achieved as described in the experiment with wet pastes (Section 5.2.2). After washing in water or isotonic solution, centrifugation and removal of the supernatant, the same procedure as described above (5.2.3a) was followed with fluidised bed drying settings of 40°C and 40 min. Moisture content determination of dried spores was also followed as described above. All treatments were done in triplicate.

(c) Screening for optimum drying protectant

Different protectants (cornmeal, cottonseed flour, starch, talc) (Sigma, UK) and at different ratios (9:1, 8:2; spores:protectant) were mixed with fresh blastospores immediately after removal of water used for washing. After that, the same procedure as described above (5.2.3a) was followed with fluidised bed drying settings of 40°C and 40 min. Moisture content determination of dried spores was also followed as described above. All treatments were done in triplicate.

5.2.4 Freeze-drying

Spore production and drying

The same blastospore treatments tested for storage stability as wet pastes were also tested for freeze-drying tolerance and subsequent stability in storage. The effect of the same post-harvest washing treatments as described above, except the glucose solution, on freeze-drying tolerance and subsequent stability in storage, was also examined. The glucose isotonic post-harvest washing treatment was not included in this study because it resulted in rapid loss of viability in storage as wet pastes (2 days after storage). In this study the best conidial treatment (see Table 4.1) was also included. The conidial treatment was produced and harvested as described in sections 2.2.4 and 2.2.5.

Initially, 4 different protective media (water, 10% skimmed milk, 10% glucose, 10% sucrose) were tested for their efficiency after thawing and freeze-drying and 2 re-hydration media (water, 10% skimmed milk) were tested for their efficiency upon re-hydration after freeze-drying. Blastospores used for this screening procedure were produced in the liquid treatment modified to 0.98 a_w using KCl and pH 8. This treatment was selected as it supported good blastospore yield, providing enough material to work with. Blastospores were harvested as described in Section 3.2.3. After harvest and removal of the spent medium, blastospores were washed in 10 ml sterile distilled water, centrifuged at 2700 r.p.m for 10 min, the supernatant removed, and the spores re-suspended in 50 ml of a sterile protective medium. A 0.5 ml aliquot from a suspension was placed in a glass vial (15 x 48 cm) and sealed with parafilm. Each protective medium was tested in triplicate. Three glass vials per replicate were prepared. All vials were placed in a freezer set at -20°C and left for 24 h. One vial from each replicate was used to test the viability of blastspores after thawing. Viability of blastospores after thawing was checked by staining the spores with viablue and examining them under fluorescent microscopy. Fluorescence was restricted to a bright outline (halo) in the case of live spores, whereas dead cells fluoresced brightly throughout. This rapid method of estimation of viability has been described for yeast cells by Hutcheson *et al.* (1988) and was evaluated for *M. anisopliae* blastospores and conidia showing excellent validity for this type of cells. The other two vials from each replicate were placed in the freeze-dryer immediately after they had been removed from

the freezer and after 3 holes had been made in the parafilm seal using a needle. The samples were left in the freeze-dryer overnight. After freeze-drying the samples were re-hydrated in either 0.5 ml of distilled water or in 0.5 ml of 10% skimmed milk. Viability after freeze-drying was evaluated using again the viablue method.

The best thawing and freeze-drying tolerance was achieved by re-suspending the washed blastospores in 10% glucose solution. Re-hydration of dried spores in 10% skimmed milk resulted in higher viability compared to re-suspension in water. These protective and rehydration solutions were used for the modified best blastospore treatments that had been subjected to hypo-osmotic shock after harvest by washing them with water, as well as for the blastospores produced from the unmodified medium and the conidial treatment. However, in order to maintain iso-osmotic conditions throughout the post-harvest washing and re-suspension in the protective solution procedure, blastospores that were washed after harvest with isotonic NaCl or PEG 200 solution, were re-suspended in 10% glucose + 1.6% NaCl (0.98 a_w) or 10% glucose + 8% PEG (0.98 a_w) respectively. Re-hydration after freeze-drying was done in 10% skimmed milk. All treatments were freeze-dried following the same procedure as described above. Each treatment was performed in triplicate and enough glass vials were made up for subsequent stability studies after storage at 4°C and 25°C for 12 weeks.

Germination studies

Storage stability was determined following the same procedure as described for wet pastes, except that germination media were inoculated with 100 µl of a spore suspension made up by adding 0.5 ml of 10% skimmed milk in a vial containing the freeze-dried spores. Germination was assessed at time 0, and after 2 and 12 weeks storage.

5.2.5 Statistical analysis

As described in Section 2.2.9

5.3 RESULTS

5.3.1 Effect of post-harvest washing treatment on stability of modified *M. anisopliae* blastospores as wet pastes

Viability of all blastospore treatments, regardless of post-harvest washing treatment, was lost rapidly in storage at 25°C and no germination was observed on water agar medium with either fully available water or with imposed water-stress (0.96 a_w) after 2 weeks storage. The same occurred at 4°C for blastospores produced under the CF+YE nitrogen profile, regardless of the solute employed for a_w modification. Only blastospores produced under the CS+YE nitrogen profile survived in storage longer than 2 weeks at 4°C as wet pastes. Results shown in Figure 5.1 and Tables 5.1 and 5.2 are for the CS+YE/NaCl treatment but the CS+YE/KCl treatment produced results with the same trends.

Figure 5.1 and Tables 5.1 and 5.2 show that iso-osmotic post-harvest washing with NaCl and PEG 200 solution resulted in improved storage stability than post-harvest washing with water. The improvement in storage stability with NaCl washing was particularly higher especially after 2 weeks storage. Additionally, only this post-harvest washing treatment resulted in improved storage stability compared to unmodified blastospores (produced under non water-stress conditions). It is interesting to note that the better storage stability of the NaCl-washed modified blastospores after 12 weeks of storage was observed only when germination was tested under water-stress conditions (Figure 5.1b). Iso-osmotic washing with glucose solution resulted in a rapid loss of viability after 2 weeks of storage.

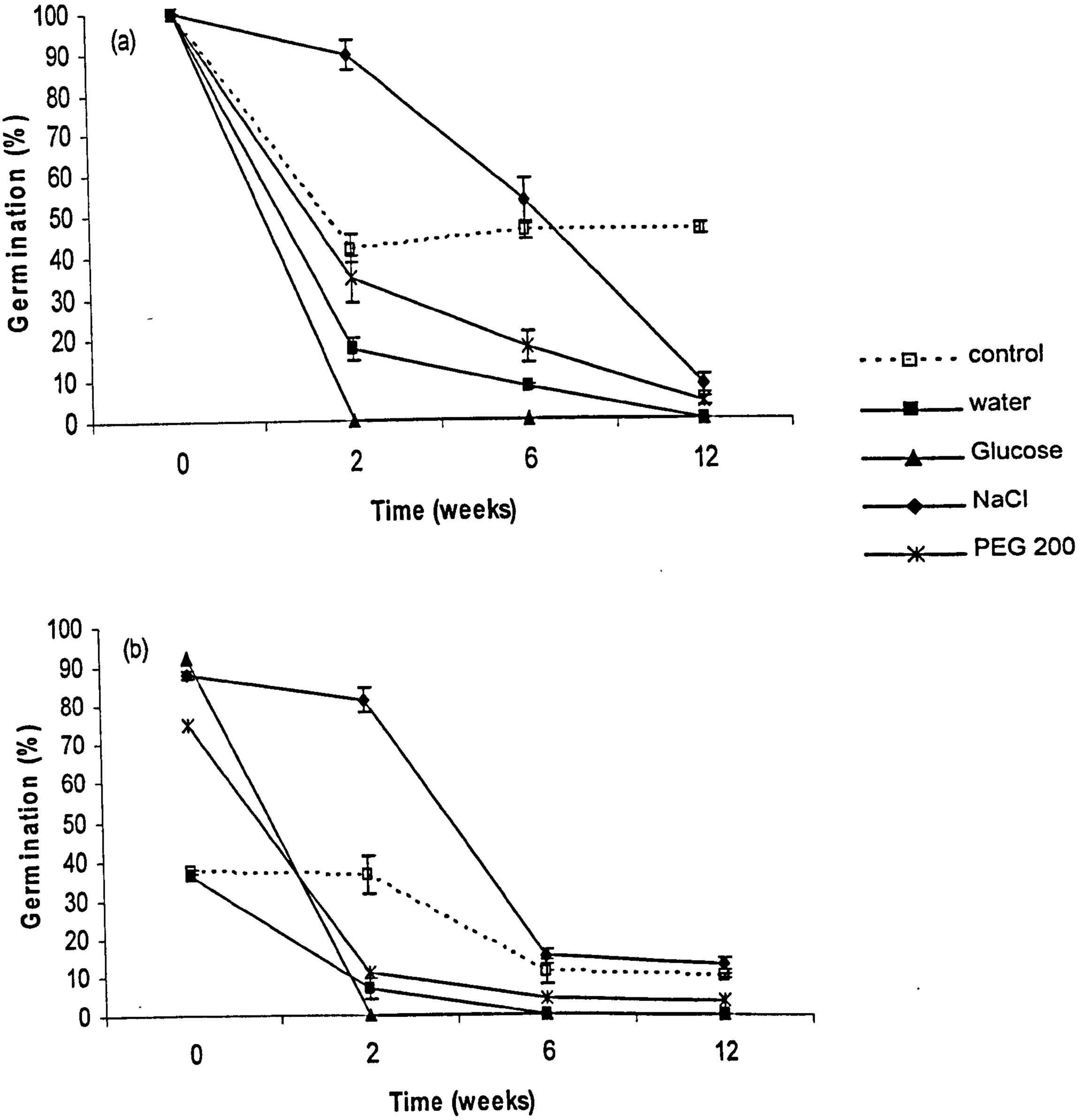


Figure 5.1. Effect of post-harvest washing treatment on stability of modified *M. anisopliae* blastospores in storage at 4°C as wet pastes. Post-harvest washing treatment was done with either water or with solutions isotonic to the growth medium with either glucose, NaCl or PEG 200. Blastospores produced from unmodified liquid medium were used as the control treatment. Stability (% germination) was evaluated (a) on water agar with fully available water (0.998 a_w) after 12 h incubation at 25°C and (b) on water agar modified to 0.96 a_w after 84 h incubation at 25°C. All data are means of three replicates per treatment. Bars represent standard errors of the means.

Table 5.1. Effect of post-harvest washing treatment on germ tube length of *M. anisopliae* blastospores stored at 4°C as wet pastes. Germ tube length (μm) was evaluated after 12 h of incubation at 25°C on water agar with fully available water (0.998 a_w).

Blastospores were harvested from modified to 0.98 a_w liquid medium (4% glucose, 3% cornsteep solid, 4% yeast extract, 1.5% NaCl) and at 72 h incubation. Post-harvest washing treatment was done with either water or with solutions isotonic to the growth medium with either glucose, NaCl or PEG 200. Blastospores produced from unmodified liquid medium were used as the control treatment. Values are means of 3 replicates ± standard error.

Washing treatment	Storage time (weeks)... 0	2	6	12
Control	+	51.61 ± 1.07	40.38 ± 0.81	39.8 ± 1.18
Water	+	34.95 ± 0.33	32.49 ± 0.66	0 ± 0
Glucose	+	0 ± 0	0 ± 0	0 ± 0
NaCl	+	72.88 ± 0.82	44.45 ± 0.68	31.55 ± 0.88
PEG 200	+	34.58 ± 1.63	33.59 ± 0.58	26.03 ± 1.02

+ Germination was too extensive to allow any germ tube length measurements

Table 5.2. Effect of post-harvest washing treatment on germ tube length of *M. anisopliae* blastospores stored at 4°C as wet pastes. Germ tube length (μm) was evaluated after 84 h of incubation at 25°C on water agar modified to 0.96 a_w.

Blastospores were harvested from modified to 0.98 a_w liquid medium (4% glucose, 3% cornsteep solid, 4% yeast extract, 1.5% NaCl) and at 72 h incubation. Post-harvest washing treatment was done with either water or with solutions isotonic to the growth medium with either glucose, NaCl or PEG 200. Blastospores produced from unmodified liquid medium were used as the control treatment. Values are means of 3 replicates ± standard error.

Washing treatment	Storage time (weeks)... 0	2	6	12
Control	28.20 ± 0.44	24.02 ± 0.81	26.60 ± 0.72	26.01 ± 1.39
Water	33.77 ± 0.38	15.71 ± 0.51	0 ± 0	0 ± 0
Glucose	61.73 ± 1.3	0 ± 0	0 ± 0	0 ± 0
NaCl	53.03 ± 0.97	30.42 ± 0.84	31.10 ± 0.66	30.33 ± 0.48
PEG 200	47.63 ± 1.58	26.07 ± 0.57	25.63 ± 1.08	23.11 ± 0.95

5.3.2 Fluidised bed drying

(a) Screening for optimum drying temperature and time

Figure 5.2 shows the effect of temperature and duration of drying on viability and final moisture content of *M. anisopliae* blastospores using a fluidised bed dryer. Viability decreased with increasing temperature and duration of drying. Interestingly, for 10 mins duration of drying, moisture content of dried blastospores did not decrease in the temperature range between 30-60°C. Although there was no noticeable change in moisture content of dried blastospores in the drying temperature range between 40-60°C and for 20 mins duration of drying, viability was dramatically decreased with increasing temperature in this range.

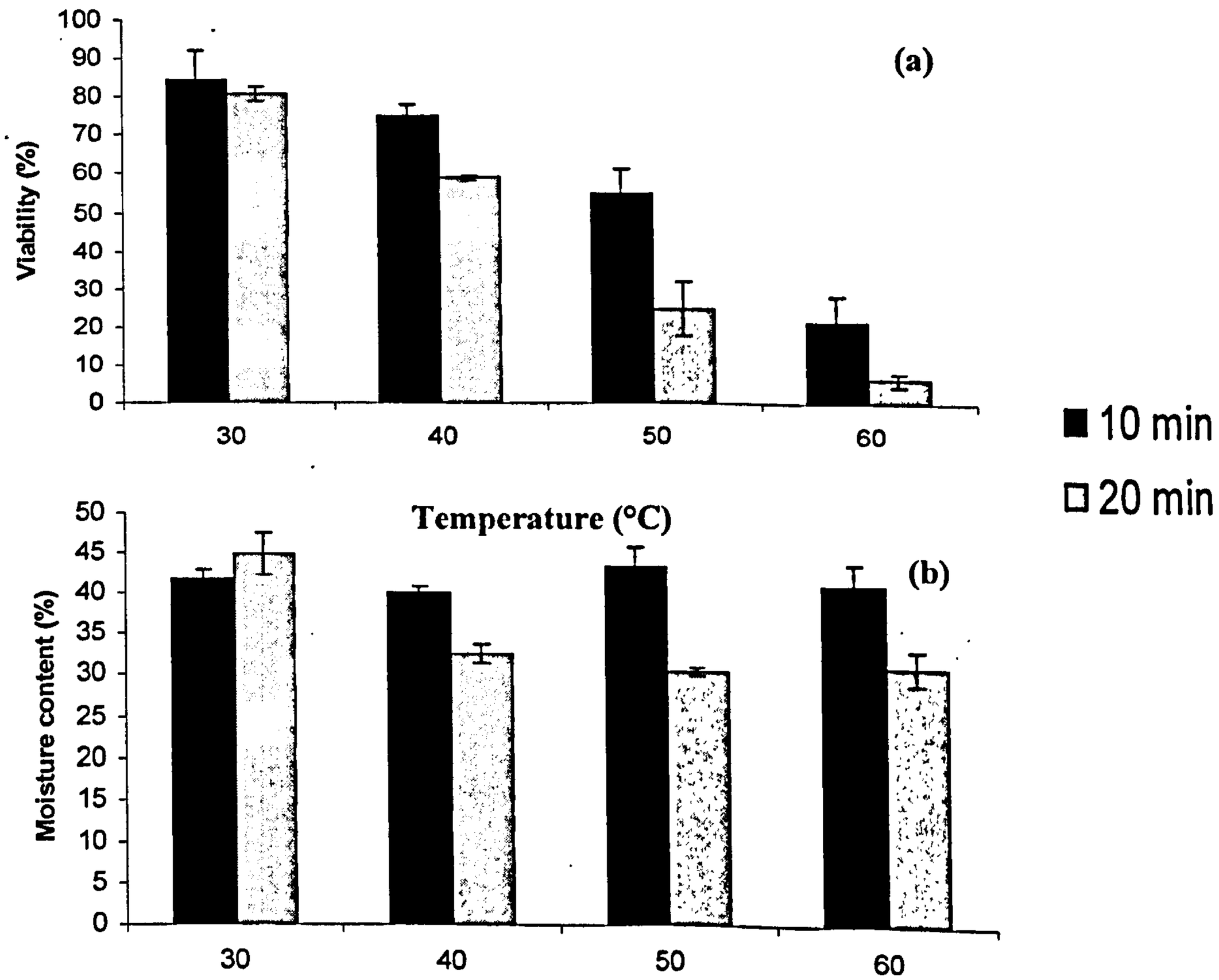


Figure 5.2. Effect of drying temperature and duration on *M. anisopliae* blastospore (a) viability and (b) final moisture content using a fluidised bed dryer. All data are means of three replicates per treatment. Bars represent standard errors of the means.

(b) Effect of osmoprotection on drying tolerance

Isotonic washing with glucose, NaCl or PEG 200 solutions resulted in a decrease in viability compared to washing with water (Figure 5.3a). Washing with glucose and NaCl in particular, dramatically reduced viability and resulted in a sticky appearance of the dried spores. Washing with glucose or PEG 200 solutions did not result in a decrease in final moisture content of dried blastospores compared to washing with water (Figure 5.3b).

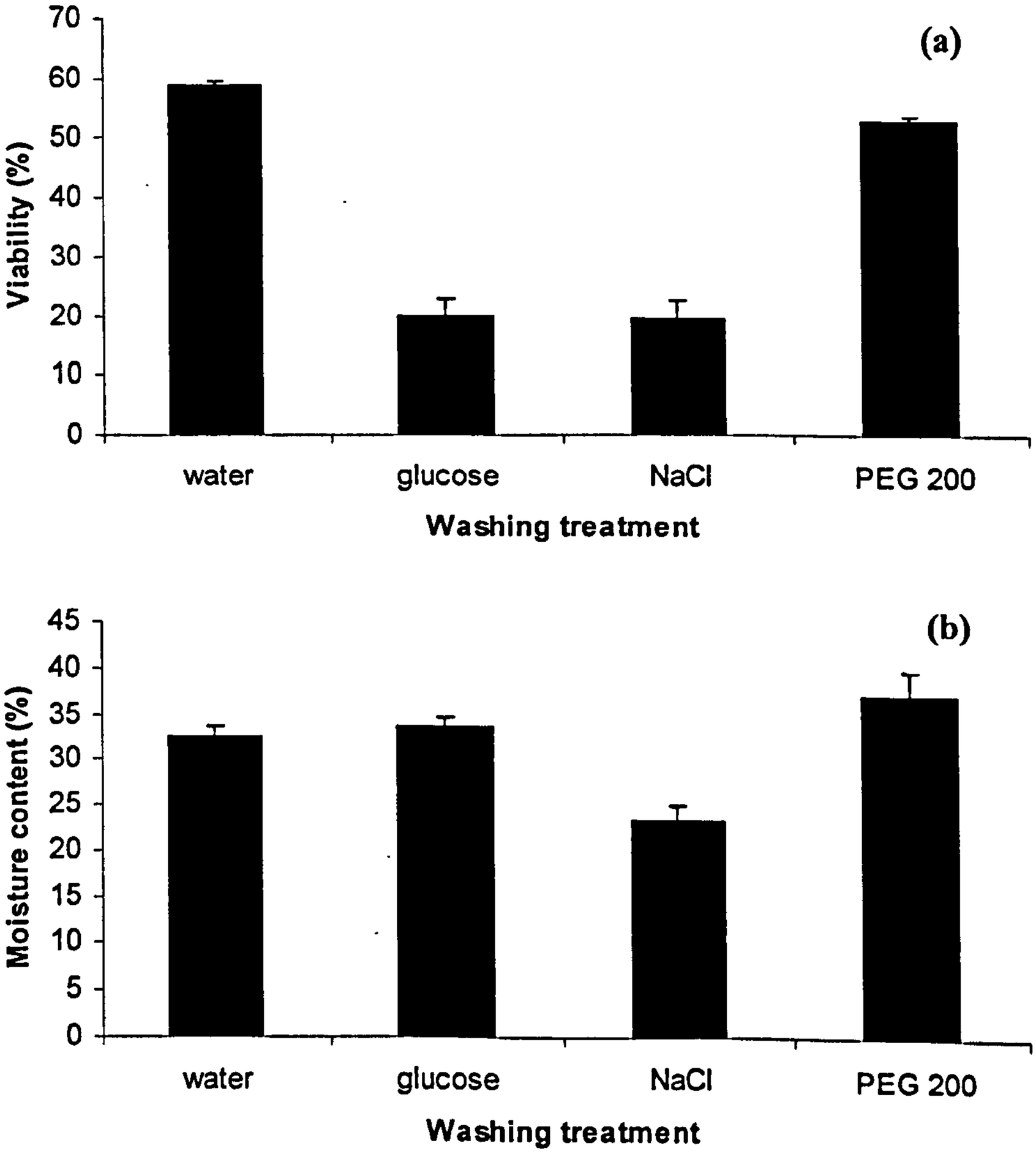


Figure 5.3. Effect of post harvest washing treatment on *M. anisopliae* blastospore (a) viability and (b) final moisture content after drying at 40°C and for 20 min using a fluidised bed dryer. Post-harvest washing treatment was done with either water or with solutions isotonic to the growth medium with either glucose, NaCl or PEG 200. Bars represent standard errors of the means.

(c) Screening for optimum drying protectant

Viability of blastospores after fluidised bed drying at 40°C and for 20 mins was higher without the use of the protectants tested in this study (Figure 5.4a). The use of protectants resulted in a decrease in final moisture content of dried blastospores compared to drying without a protectant, except when cottonseed flour was used at 9:1 spores:protectant ratio (Figure 5.4b). As in the case with drying temperature, drying duration and washing optimisation, a drop in viability was not always concomitant with a drop in final moisture content of the dried spores (when cottonseed flour was used at 9:1).

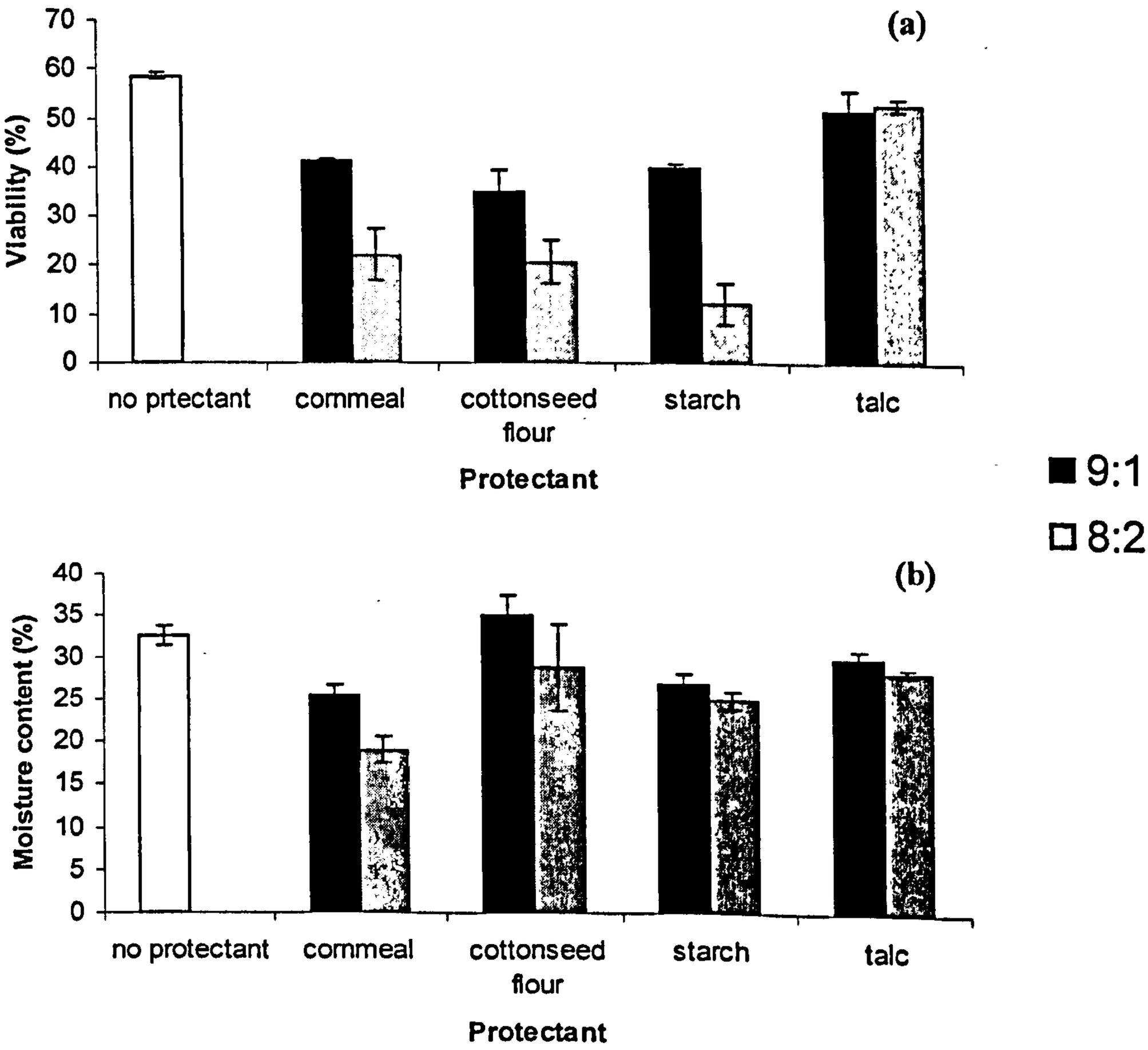


Figure 5.4. Effect of type and ratio (parts of spores:parts of protectant) of protectant on *M. anisopliae* blastospore (a) viability and (b) final moisture content after drying at 40°C and for 20 mins using a fluidised bed dryer. Bars represent standard errors of the means.

5.3.3 Effect of protective solutions and rehydration medium on *M. anisopliae* blastospore freeze-drying tolerance

Before studying the effect of post-harvest washing treatment on freeze-drying tolerance and subsequent stability of modified *M. anisopliae* blastospores, 4 different protective media (water, 10% skimmed milk, 10% glucose, 10% sucrose) and 2 re-hydration media (water, 10% skimmed milk) were tested for their protection efficiency after thawing and freeze-drying and upon re-hydration after freeze-drying repsectively. Best thawing protection was conferred by either 10% skimmed milk or 10% glucose (Figure 5.5). Best freeze-drying protection was conferred by 10% glucose followed by 10% sucrose (Figure 5.6). Regardless of protective medium, re-hydration in 10% skimmed milk resulted in higher viability than re-hydration in water. Therefore, for subsequent studies 10% glucose was used as the protective medium and 10% skimmed milk as the re-hydration medium.

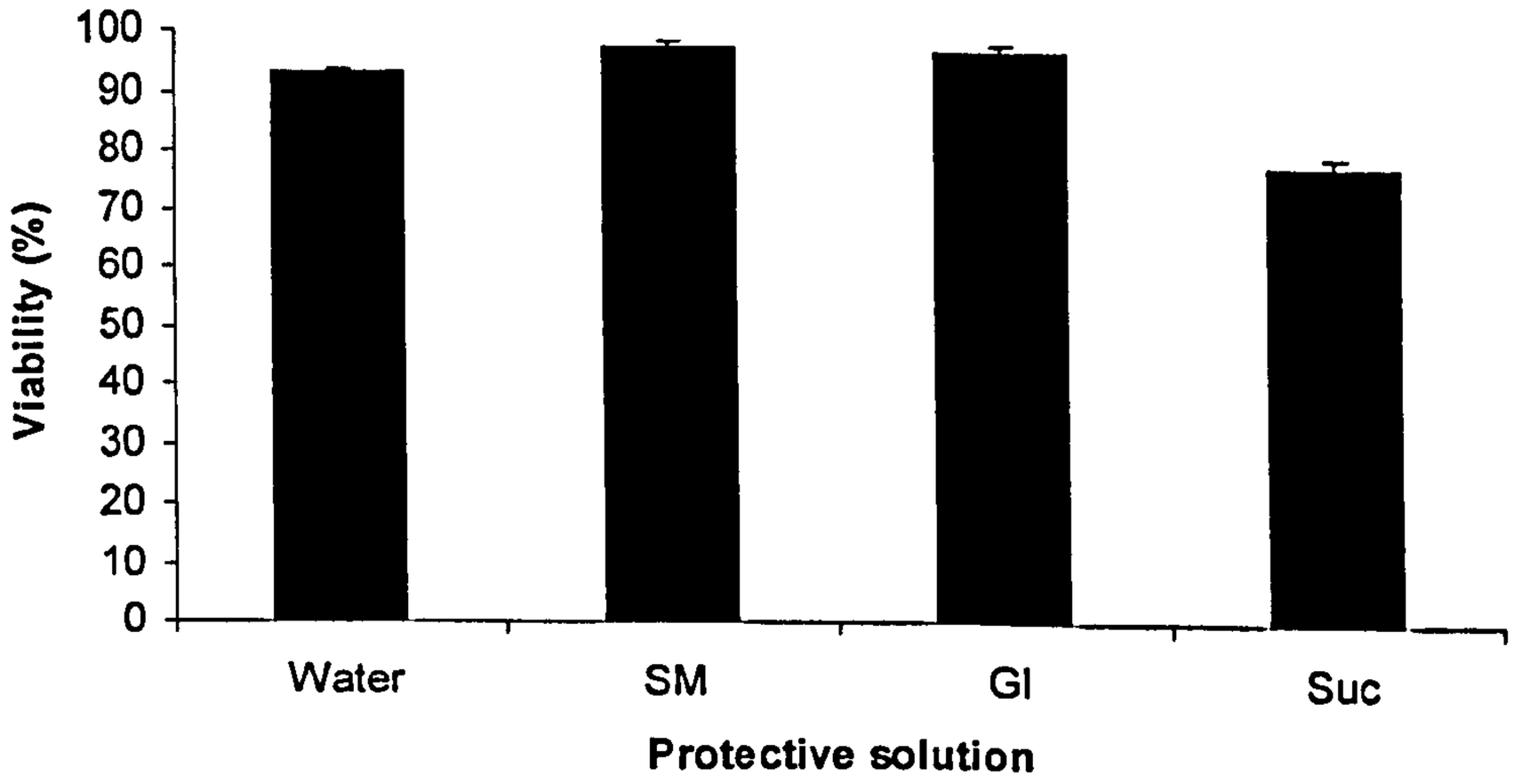


Figure 5.5. Effect of protective solutions on *M. anisopliae* blastospore freeze tolerance. SM: 10% skimmed milk; Gl: 10% glucose; Suc: 10% sucrose. All data are means of three replicates per treatment. Bars represent standard errors of the means.

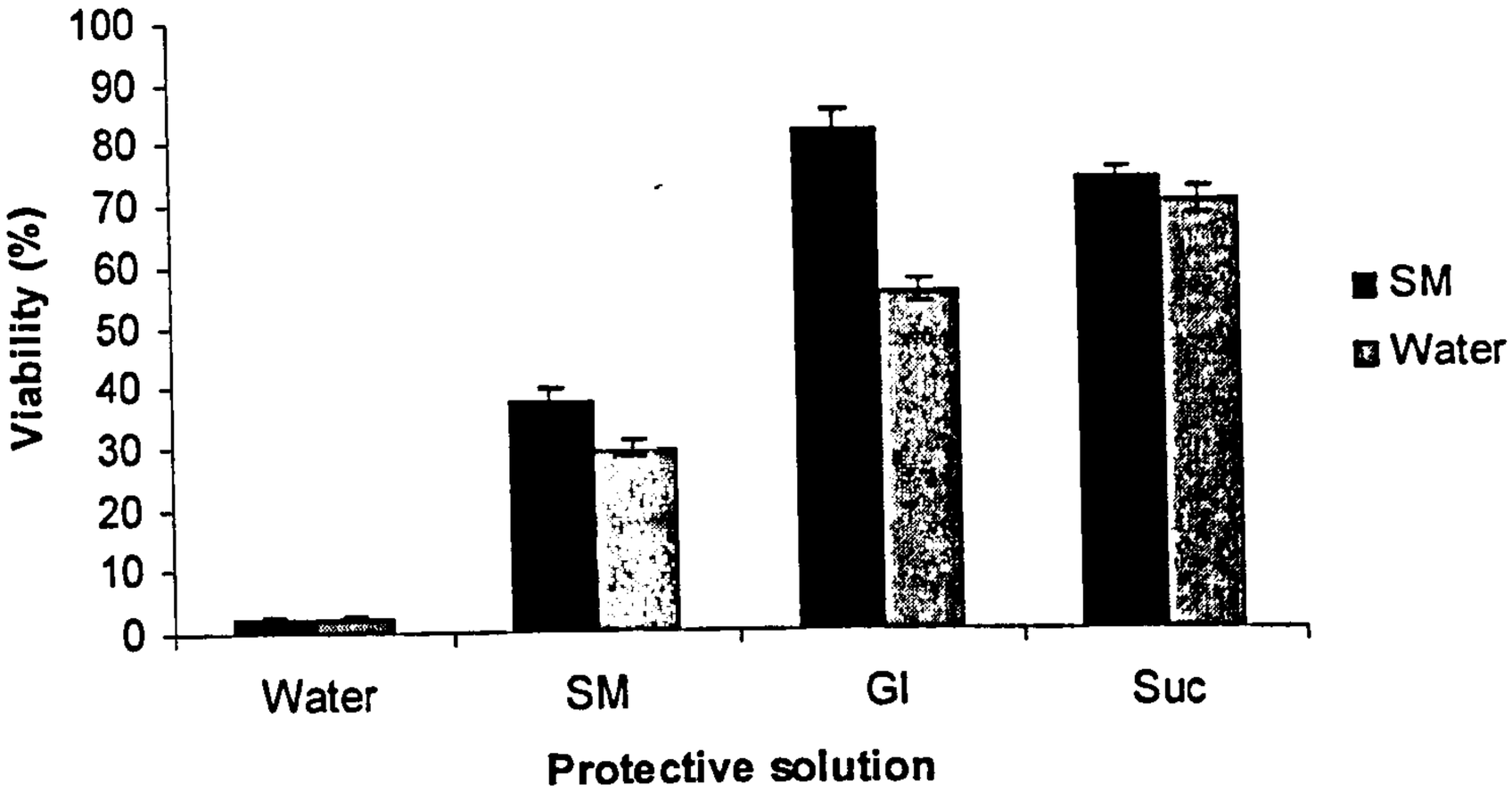


Figure 5.6. Effect of protective solutions (water; SM: 10% skimmed milk; Gl: 10% glucose; Suc: 10% sucrose) and rehydration medium (10% skimmed milk ■, water ◘) on *M. anisopliae* blastospore freeze-drying tolerance. All data are means of three replicates per treatment. Bars represent standard errors of the means.

5.3.4 Effect of post harvest washing treatment on freeze-drying tolerance and subsequent storage stability of modified *M. anisopliae* propagules

Freeze-drying of modified blastospores that had been washed in isotonic PEG 200 solution after harvest resulted in 100% mortality of the cells. Post-harvest washing of modified blastospores with water or isotonic NaCl solution did not influence freeze-drying tolerance and viability after treatment. In both cases viability was very high (>92%; data not shown). Viability after freeze-drying was also very high (>92%) for the unmodified blastospore treatment and for the conidial treatment. However, post-harvest washing treatment of modified blastospores affected germination efficiency immediately after freeze-drying and subsequent storage stability. When germination was tested immediately after freeze-drying on water agar with fully available water, all blastospore treatments, regardless of post-harvest washing treatment, gave very high germination (>95%) (Figure 5.7a) and germ tube length was too extensive after 12 h incubation to allow measurements. Freeze-dried modified conidia germinated slower under the same conditions (Figure 5.7a). When germination was tested on water agar medium with imposed water-stress immediately after freeze-drying, modified

blastospores that had been washed after harvest with isotonic NaCl solution germinated better than blastospores that had been washed with water (Figure 5.7b).

Enhancement of germination due to isotonic post-harvest washing treatment was significant ($P<0.05$) after 2 weeks storage for both fully available or stressed water conditions (Figure 5.8). Germ tube extension was also remarkably enhanced by isotonic post-harvest washing treatment, except for the CS+KCl treatment (Tables 5.3 and 5.4). In contrast to wet pastes, blastospores that were produced under the CF+YE nitrogen profile and modified with either KCl or NaCl showed better stability as freeze-dried spores compared to blastospores that were produced under the CS+YE nitrogen profile and under the corresponding modifying solute (KCl, NaCl). It is interesting to note that after 2 weeks storage unmodified blastospores (control treatment) retained significantly ($P<0.05$) higher germinability under water-stress conditions than modified blastospores that had been washed with water after harvest (Figure 5.8). Modified conidia showed the lowest germinability after two weeks of storage compared to all blastospore treatments, except for the CS+KCl treatment which did not germinate at all after 2 weeks of storage. None of the treatments germinated after 12 weeks of storage.

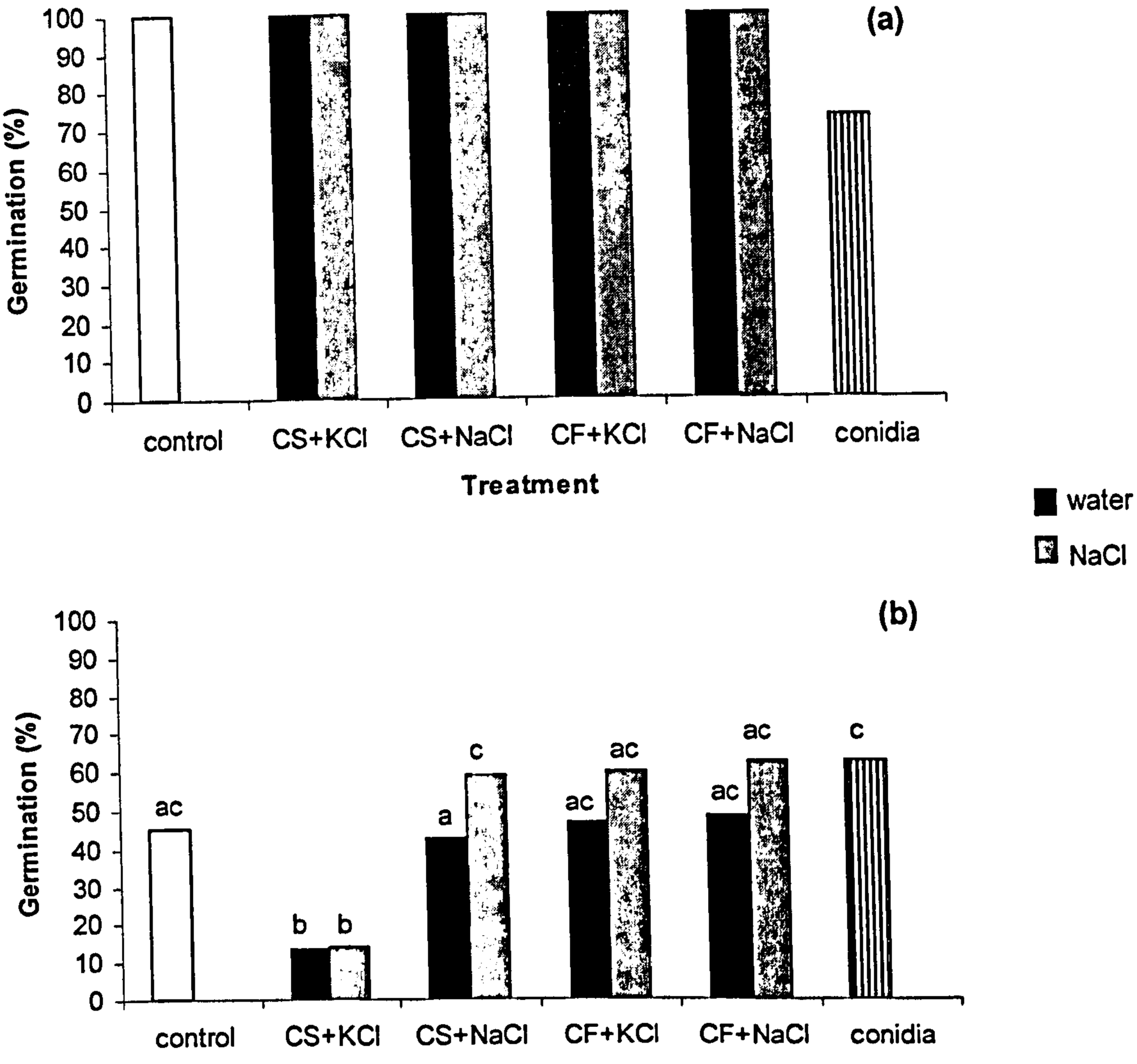


Figure 5.7. Effect of post-harvest washing treatment on germinability of *M. anisopliae* freeze-dried blastospores immediately after drying, (a) under conditions with fully available water (0.998 a_w) and (b) under water-stress conditions (0.96 a_w). Germination was evaluated after 12 h incubation at 25°C on water agar with fully available water and after 84 h incubation at 25°C on water agar modified to 0.96 a_w with PEG 200. Modified blastospores were harvested at 72 h incubation from modified to 0.98 a_w (using KCl or NaCl) liquid medium with either cornsteep solid (CS) or cottonseed flour (CF) in combination with yeast extract as the nitrogen source. Conidia were produced on modified to 0.98 a_w (using water alone) bulgar wheat. Post-harvest washing treatment was done with either water or with NaCl solution isotonic to the growth medium. All data are means of three replicates per treatment. Different letters indicate statistical differences ($P<0.05$) between means.

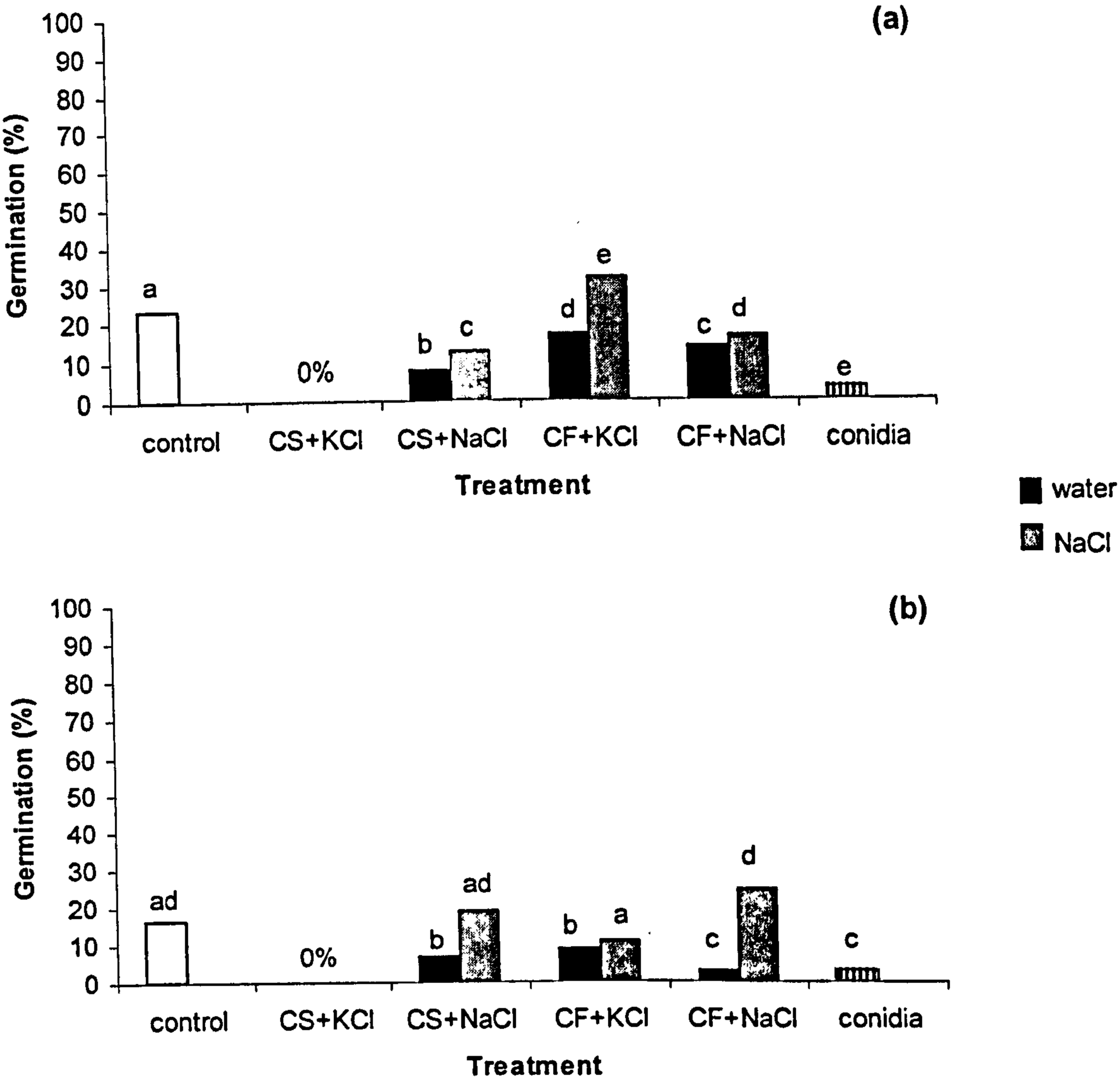


Figure 5.8. Effect of post-harvest washing treatment on germinability of *M. anisopliae* freeze-dried blastospores after 2 weeks in storage at 4°C (a) under conditions with fully available water (0.998 a_w) and (b) under water-stress conditions (0.96 a_w). Germination was evaluated after 12 h of incubation at 25°C on water agar with fully available water and after 84 h of incubation at 25°C on water agar modified to 0.96 a_w with PEG 200. Modified blastospores were harvested at 72 h of incubation from modified to 0.98 a_w (using KCl or NaCl) liquid medium with either cornsteep solid (CS) or cottonseed flour (CF) in combination with yeast extract as the nitrogen source. Conidia were produced on modified to 0.98 a_w (using water alone) bulgar wheat. Post-harvest washing treatment was done with either water or with NaCl solution isotonic to the growth medium solution. All data are means of three replicates per treatment. Different letters indicate statistical differences ($P<0.05$) between means.

Table 5.3. Effect of post-harvest washing treatment on germ tube extension of modified *M. anisopliae* freeze-dried blastospores in storage at 4°C. Germ tube length (μm) was evaluated after 12 h incubation on water agar medium with fully available water (0.998 a_w).

Storage time (weeks)...	**0**		**2**	
	Water	**NaCl**	**Water**	**NaCl**
Control[a]	+	++	78.09 ± 1.35	++
CS+KCl[b]	+	+	0 ± 0	0 ± 0
CS+NaCl[b]	+	+	20.18 ± 0.66	35.27 ±1.64
CF+KCl[b]	+	+	97.73 ± 0.93	154.51 ± 6.51
CF+NaCl[b]	+	+	70.72 ± 0.94	87.58 ± 1.04
Conidia[c]	62.27 ± 1.77	++	14.47 ± 0.52	++

[a] Blastospores produced in unmodified liquid medium

[b] Modified blastospores were produced in modified to 0.98 a_w (using KCl or NaCl) liquid medium with either cornsteep solid (CS) or cottonseed flour (CF) in combination with yeast extract as the nitrogen source.

[c] modified conidia were produced on modified to 0.98 a_w (using water alone) bulgar wheat

+ Too extensive germination to allow any measurements

++ No isotonic washing treatment

Post-harvest washing treatment was done with either water or with isotonic to the growth medium solutions made up with either glucose, NaCl or PEG 200.

Values are means of 3 replicates ± standard error

Table 5.4. Effect of post-harvest washing treatment on germ tube extension of modified *M. anisopliae* freeze-dried blastospores in storage at 4°C. Germ tube length (μm) was evaluated after 84 h incubation on water agar medium with imposed water-stress (0.96 a_w).

Storage time (weeks)...	**0**		**2**	
	Water	**NaCl**	**Water**	**NaCl**
Control	154 ± 3.61	++	141.93 ± 2.02	++
CS+KCl	67.80 ± 1.73	58.47 ± 3.47	0 ± 0	0 ± 0
CS+NaCl	94.00 ± 1.42	133.17 ± 5.49	80.45 ± 0.87	107.56 ± 4.41
CF+KCl	65.47 ± 0.69	84.93 ± 1.66	60.17 ± 0.42	80.84 ± 0.72
CF+NaCl	88.97 ± 1.00	97.10 ± 1.15	79.33 ± 0.88	82.87 ± 1.99
Conidia	56.27 ± 2.50	++	41 ± 0.64	++

Subheadings same as in Table 5.3

5.4 DISCUSSION

This study considered the effect of cultural conditions and osmoprotection during harvest of modified *M. anisopliae* propagules on their stability in storage as wet pastes and freeze-dried spores as well as on freeze-drying tolerance. Fluidised bed drying was not considered for subsequent studies because during a number of optimisation steps it was either highly detrimental to spore viability or was not efficient at reducing the final moisture content below 30%. Moisture percentage of dried fungal spores must be lower than 10% in order to be good candidates for increased storage stability (Moore *et al.*, 1996). The effect of osmoprotection of blastospores was however examined because the treatment used for the screening process was a modified one. Washing with isotonic solutions (glucose, NaCl, PEG 200) resulted in a decrease in viability of fluidised bed dried blastospores. It is likely that the compounds used to make isotonic solutions are detrimental to *M. anisopliae* blastospores during this type of drying. No conclusions, therefore, can be drawn whether retention of higher endogenous reserves by osmoprotection of modified *M. anisopliae* blastospores results in increased drying resistance using this method.

Stability in storage as wet pastes or freeze-dried spores was greatly influenced by the type of post-harvest washing treatment (iso-osmotic, water) as well as by the nitrogen nutritional status of the liquid production medium. Regardless of post-harvest washing treatment, modified blastospores produced under the CF+YE nitrogen source profile completely lost their germinability after 2 weeks storage as wet pastes (at both 25 and 4°C) (data not shown) and only blastospores produced under the CS+YE nitrogen source treatment (unmodified and modified) retained their germinability after 2 weeks storage at 4°C. However, viability of the CF+YE nitrogen treatments was retained after 2 weeks storage at 4°C as freeze-dried spores with germination efficiency comparable or even better than blastospores from the CS+YE nitrogen source treatments. Modified blastospores produced under the CF+YE nitrogen source treatment and at pH 8 contained higher amounts of endogenous protein (Figure 3.9) and of the polyols erythritol and mannitol (Figure 3.7) than blastospores produced under the CS+YE nitrogen source status. Germination of the former fresh blastospore treatments was also significantly ($P<0.05$) higher than blastospores from the latter treatment (Figure 3.10). It

is therefore very likely that the higher metabolic activity of the blastospores produced under the CF+YE nitrogen source treatment leads to a rapid loss of their viability when stored as wet pastes, whereas when metabolic activity is slowed down by freeze-drying, the higher content of endogenous reserves of these treatments prolongs their stability in storage. Improved survival in storage of freeze-dried fungal spores with increased levels of polyols has been reported before for *E. nigrum* (Pascual *et al.*, 2003). Two recent studies have considered the effect of nitrogen source on the stability of dried fungal BCAs, but they did not provide any information about the endogenous composition of the spores produced under different environmental regimes (Sandoval-Coronado *et al.*, 2001; Jackson *et al.*, 2003).

Post-harvest washing of modified *M. anisopliae* blastospores from the CS+YE nitrogen source treatment, greatly influenced their stability as wet pastes. Regardless of the liquid culture modifying solute, post-harvest osmoprotection of modified blastospores using isotonic NaCl or PEG 200 solutions resulted in an improved storage stability compared to washing with water. The improvement in storage stability was most pronounced with the NaCl isotonic solution resulting in up to more than 70% difference in germination after 2 weeks storage compared to washing with water, whether germination was checked on media with fully available water or with imposed water-stress. Post-harvest osmoprotection of modified *M. anisopliae* blastospores resulted in retention of the accumulated endogenous polyols during culture, whereas washing with water led to a leakage of these reserves (Figures 3.2 and 3.3). Since PEG 200 is inert and is not utilised by fungi as a nutritional source (Inch & Trinci, 1987; Humphreys *et al.*, 1989), the improvement in stability as wet pastes conferred by this isotonic washing should be due to osmoprotection and subsequent retention of the endogenous reserves in blastospores rather than to an interference with spore germination. However, whether the remarkable improvement of stability conferred by the NaCl isotonic washing treatment is entirely due to osmoprotection is not clear from the present study and the effect of NaCl on blastospore germination should be more closely examined. One interesting observation is that after 12 weeks storage and when germination was checked under conditions with fully available water, the control treatment (blastospores produced without conditions of imposed water-stress) had a significant ($P<0.05$) higher

percentage germination compared to all modified and osmoprotected blastospore treatments, whereas under water-stress conditions spores from the NaCl osmoprotected modified blastospore treatment germinated better than the control treatment. This shows that although osmoprotected modified blastospores lost their stability in storage faster than unmodified blastospores, the ones that survived retained better osmoregulation ability. The fact that osmoprotected modified blastospores using either NaCl or PEG 200 isotonic solution germinated better on water agar with fully available water compared to modified blastospores washed with water, shows a contribution of osmoprotection to increased stability in storage, as under such conditions osmoregulation may not be as important as under conditions of limited water availability. More studies need to be done to determine whether the endogenous retention of polyols is responsible for an enhanced stability in storage or for retention of osmoregulation.

Interestingly, isotonic washing treatment with glucose solution resulted in a rapid loss of germination after 2 weeks of storage as wet pastes for all modified blastospore treatments. One possible explanation could be that the presence of glucose could enhance metabolic activity of the spores leading to a rapid loss of stability in storage. This phenomenon should be more closely examined.

It is now widely accepted that re-suspension of fungal spores in sugar solutions, mainly disaccharides, confers desiccation tolerance and enhanced survival upon re-hydration. The proposed mechanism of desiccation protection by sugars is known as the "water replacement hypothesis", whereby the sugars replace water molecules in the membranes and form hydrogen bonds with the phospholipids, thus preventing collapse of the membrane upon water removal (Crowe *et al.*, 1987). The net result of this is preservation of the liquid-crystalline state of the membrane after drying, which means that upon re-hydration, the membrane does not go through a phase transition from the gel state (drying without sugars and therefore tight packing of phospholipids polar headgroups) to the liquid-crystalline state. Such a transition may result in leakage of internal solutes upon re-hydration (Crowe & Crowe, 1986). Therefore, re-suspension of fungal propagules in sugar solutions before drying can protect them during the drying

procedure and upon subsequent re-hydration. Leakage of internal solutes however may occur, as has already been described, during the harvest step where some studies re-suspend fungal propagules in water for spent medium removal in order to remove compounds that might be detrimental upon drying or might interfere with subsequent studies (e.g. Jin *et al.* 1991; Costa *et al.*, 2000). This causes osmotic stress and loss of internal solutes. In the present study it was found that osmoprotection of modified *M. anisopliae* blastospores after harvest resulted in increased germination efficiency immediately after freeze-drying and after 2 weeks storage, confirming the hypothesis that the loss of endogenous reserves upon osmotic shock may result in a subsequent reduction in stability. Montazeri & Greaves (2002) also found that washing of *C. truncatum* conidia with sucrose or NaCl solutions before air-drying resulted in increased storage stability compared to unwashed conidia but whether this was an osmoprotection effect is difficult to determine, because how the unwashed spores were treated before drying was not detailed and because no measurements of the water potential of the production and the washing medium were made to evaluate whether iso-osmotic or osmotic shock conditions occurred during the washing procedure.

Unmodified freeze-dried blastospores retained a higher germinability both immediately after freeze-drying and after two weeks of storage compared to all modified blastospores that had been washed with water after harvest. The high osmotic shock and rapid loss of endogenous reserves that modified blastospores undergo when they are washed with water could explain this observation.

This study has showed the importance of osmoprotection of *M. anisopliae* blastospores that are produced under water-stress conditions in storage stability and osmoregulation retention. Subsequent studies should consider this effect before they subject fungal propagules to different drying regimes.

Chapter 6 BIOASSAYS

6.1 INTRODUCTION

The speed with which entomopathogenic fungi kill their insect hosts depends on a wide array of biotic and a-biotic factors. Biotic factors include speed of germination (Hassan *et al.*, 1989; Hallsworth & Magan, 1994c), enzyme production (Coudron *et al.*, 1984; Jackson *et al.*, 1985; St. Leger *et al.*, 1988a; St. Leger *et al.*, 1992), and toxin production (Pedras *et al.*, 2002). A-biotic factors include availability of nutrients (endogenous to the cuticle, exogenously deposited, or host exudates), presence or absence of inhibitory compounds to germination on the outermost surface of the cuticle and environmental factors like relative humidity, temperature, and UV radiation. The amount of spores deposited on an insect host as well as the type of spore will also affect the speed of infection. For example, the hydrophobic conidia of *M. anisopliae* and *B. bassiana* possess an outer rodlet layer which in addition to providing protection against dehydration and aiding in the aerial dispersal of conidia, may have a major role in the attachment of conidia to the host cuticle which is also hydrophobic (Boucias & Pendland, 1991).

For the above reasons it is easy to understand that improved *in vitro* germination efficiency of a fungal entomopathogen will not always translate into improved pathogenicity. It is therefore necessary that *in vitro* studies are followed by bioassays on insect hosts and subsequently by field trials. By controlling different biotic and abiotic factors in a bioassay system, it is possible to evaluate differences in pathogenicity between different spore treatments or between different fungal isolates.

In this study, the pathogenicity of the best two blastospore treatments and the best conidial treatment as well as the effect of osmoprotection of modified blastospores on their pathogenicity were evaluated with a bioassay system using *Aphis gossypii* aphids of the same age and under steady state temperature and photoperiod conditions.

6.2 MATERIALS AND METHODS

A. gossypii stock culture

Aphids were reared on marrow plants (*Cucurbita pepo*), cv. Gold Rush (Elsom Seeds Ltd, Lincoln, UK). Plants were grown in Levington compost (M2) in 7 cm pots in the glasshouse (ca. 20°C) for approximately 4 weeks (until the third true leaf had emerged). Aphid cultures were maintained in perspex cages (380 x 380 x 380 mm) in which two opposite sides are cut away (340 x 340 mm), and replaced with gauze to allow air circulation. Cages were maintained within a controlled environment room at 20 ± 2°C, photoperiod 16 h. Plants were watered 3 times weekly. Cultures were changed sequentially by removing 1 leaf from an old culture plant and placing it on top of a fresh leaf in the new culture in order to ensure a constant supply of adult aphids.

A. gossypii synchronised culture

Twenty to thirty mature apterous virginoparae were confined to a single leaf of a whole plant by enclosing a leaf blade within a clear polyethylene box (124 x 82 x 22 mm). The petiole was fitted into a hole (10 x 17 mm) cut in the base of the box. Gauze-covered ventilation holes were cut in the side of the box (40 x 40 and 40 x 90 mm) and cotton wool was wrapped around the petiole at the point of entry into the box to block any gaps through which the aphids can escape. Plastic plant identification labels are taped to the back of the box and pushed into the soil to support the plant. Virginoparae were maintained on the plants for 24 h after which they were removed and the progeny maintained for a further 9 days as above (20 ± 2°C, photoperiod 16 h, plants watered 3 times weekly).

Spore treatments

The following fungal spore treatments were evaluated:

1. Unmodified blastospores (see Section 3.2.2)
2. Modified blastospores from the CS+NaCl, 0.98 a_w, pH 8 treatment (see Section 3.2.6)
3. Modified blastospores from the CF+KCl, 0.98 a_w, pH 8 treatment (see Section 3.2.6)

4. Modified conidia from 5-day-old bulgar wheat cultures modified to 0.98 a_w (see Section 2.2).

Unmodified blastospores were washed with water and modified blastospores were washed with either water or with isotonic NaCl solution (3.55%) after harvest. Spore suspensions (10^7 spores ml^{-1}) were made in water or isotonic NaCl solutions accordingly.

Bioassay procedure

Bioassay chambers consisted of a clear polyethylene box (124 x 82 x 22 mm) with the base and the back of the box lined with damp filter paper. A single leaf of a 3-4 week-old marrow plant was enclosed within the box by fitting the petiole into a hole (10 x 17 mm) cut in the base of the box. Cotton wool was wrapped around the petiole at the point of entry into the box to block any gaps through which the aphids could escape. Plant identification labels were taped to the back of the box and pushed into the soil to provide support. Groups of 25-30 apterous, fixed-age aphids were placed on damp filter paper in 9 cm diameter Petri dish lids, and sprayed with 2 ml spore suspension using a Potter tower with an intermediate atomiser and a spray pressure of 50 kPa. Controls were sprayed with either 0.01% triton X-100 alone or with 0.01% triton X-100 + 3.55% NaCl. These were sprayed prior to any fungal application to prevent accidental infections. Sprayed aphids were maintained in Petri dishes on the bench for 1 h post-treatment. Twenty most active individuals were transferred to the bioassay chambers using a fine camel-hair paintbrush. Bioassay chambers were maintained within a controlled environment room at 20 ± 2°C, photoperiod 16 h and plants watered 3 times weekly. Filter papers were moistered on a daily basis to ensure 100% R.H. The number of alive and dead aphids was monitored every day for 7 days. Cadavers were placed on damp filter papers, sealed in Petri dishes, and examined for the appearance of conidia on the integument, 7 days after the end of the bioassay. Viability of all fungal treatments was assessed by measuring the germination of spores on water agar after incubation for 12 h at 25°C. In an attempt to estimate the mortality that was due to fungal pathogenicity only, control mortality was deducted from treatment mortality. This was referred to as pathogenesis-related mortality.

Bioassay design

A randomised block design was used: 6 reps of 5 treatments, 2 water controls, and 2 NaCl (3.55%) solution controls. Each plot consisted of 2 plants, 20 aphids per plant (n=40).

Statistical analysis

As described in Section 2.2.9

6.3 RESULTS

All presented data are pooled results from 5 repeats of bioassays. Figure 6.1 shows the mortality of aphids caused by different propagule treatments of *M. anisopliae*. Inoculation with modified conidia resulted in the highest mortality of aphids from day 3 to day 7. Mortality caused by conidia between day 3 and 4 was significantly ($P<0.05$) higher compared to all blastospore treatments. However, at the end of the experiment (day 7) modified blastsospores produced under the CF+YE nitrogen profile caused as high mortality as the conidial treatment. Treatment with 0.01% triton X-100 + NaCl caused higher mortality than treatment with 0.01% triton X-100 alone. It is very interesting to note that when control mortality was deducted from treatment mortality, there was a significant ($P<0.05$) reduction in aphid total mortality (day 7) when washing was done with isotonic NaCl solution (Figure 6.2).

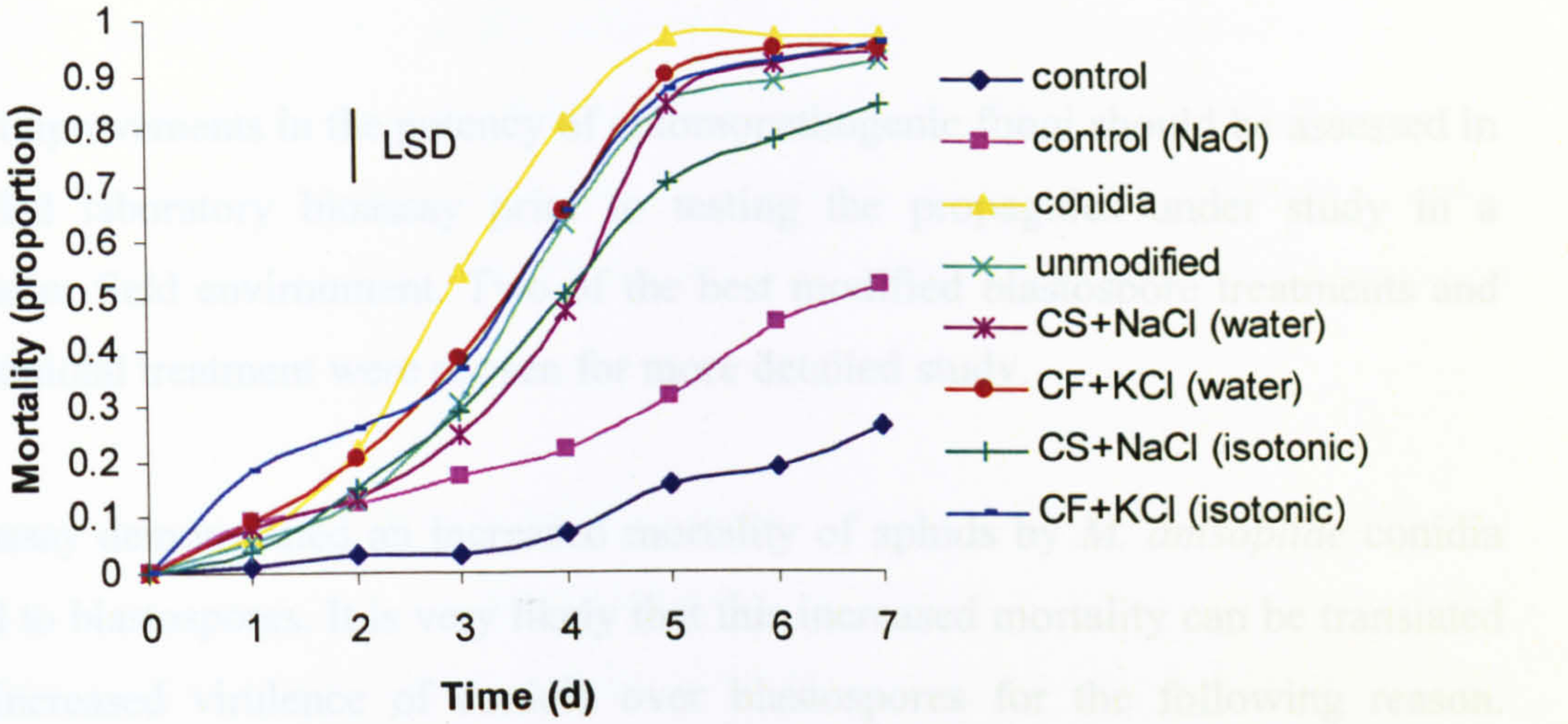


Figure 6.1. Effect of modified blastospores and conidia of *M. anisopliae* and washing treatment on temporal mortality of *A. gossypii*. Modified blastospores were harvested at 72 h of incubation from modified to 0.98 a_w (using KCl or NaCl) liquid medium with either cornsteep solid (CS) or cottonseed flour (CF) in combination with yeast extract as the nitrogen source. Conidia were produced on modified to 0.98 a_w (using water alone) bulgar wheat. Post-harvest washing treatment was done with either water or with isotonic to the growth medium solution made up with NaCl. The control treatment was 0.01% triton X-100 and the control (NaCl) was 0.01% triton X-100 + 3.55% NaCl.

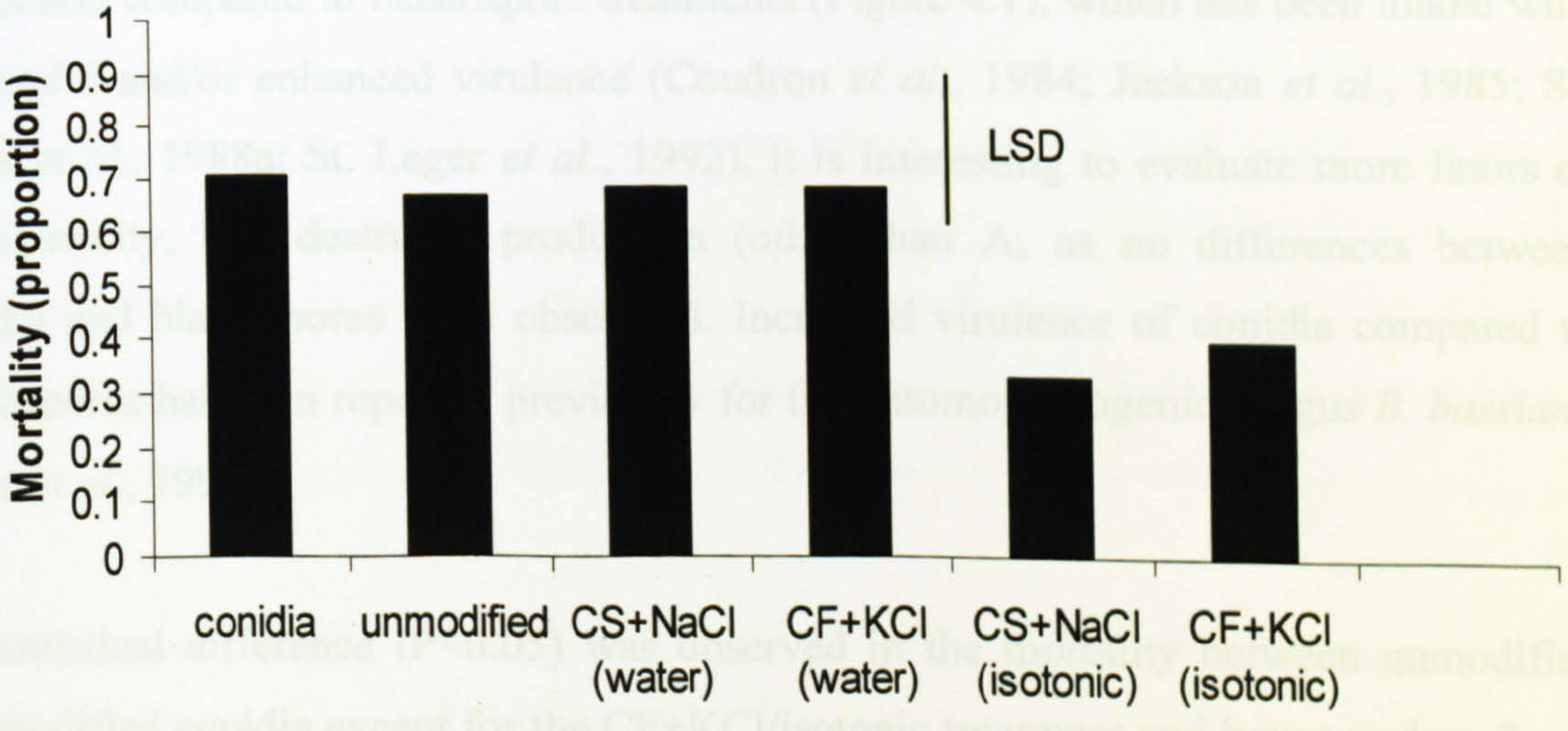


Figure 6.2. Effect of modified blastospores and conidia of *M. anisopliae* and washing treatment on pathogenesis-related total mortality (day 7) of *A. gossypii*.

6.4 DISCUSSION

Potential improvements in the potency of entomopathogenic fungi should be assessed in a controlled laboratory bioassay prior to testing the propagules under study in a glasshouse or field environment. Two of the best modified blastospore treatments and the best conidial treatment were chosen for more detailed study.

This bioassay demonstrated an increased mortality of aphids by *M. anisopliae* conidia compared to blastospores. It is very likely that this increased mortality can be translated into an increased virulence of conidia over blastospores for the following reason. Bioassays were performed under 100% R.H., conditions under which modified conidia had slower germination rates than both modified and unmodified blastospores (see Sections 2.3.7 and 3.3.5). Therefore, the observed increased mortality cannot be attributed to a faster germination rate of conidia. The hydrophobic nature of the outer layer of conidia which may result in a better attachment to the hydrophobic host cuticle compared to the hydrophilic blastospores, should have been eliminated by the use of the wetting agent (Triton X-100) in the spore suspensions. Considering that *M. anisopliae* conidia produced in bulgar wheat had a noticeable lower rate of extracellular protease production compared to blastospore treatments (Figure 4.1), which has been linked with successful and/or enhanced virulence (Coudron *et al.*, 1984; Jackson *et al.*, 1985; St. Leger *et al.*, 1988a; St. Leger *et al.*, 1992), it is interesting to evaluate more fators of pathogenicity, like destruxin production (other than A, as no differences between conidia and blastospores were observed). Increased virulence of conidia compared to blastospores has been reported previously for the entomopathogenic fungus *B. bassiana* (Lane *et al.*, 1991b).

No statistical difference ($P<0.05$) was observed in the mortality between unmodified and modified conidia except for the CF+KCl/isotonic treatment and between days 2 and 3. However, care must be taken when interpreting results from bioassays without examining whether the mortality is due to pathogenicity or due to handling procedures and/or to the effect of any additives that are used in the spore suspensions. It is possible, therefore, that the aforementioned statistical difference is due to an adding mortality

caused by the isotonic solution itself. This is highly suspected as the control treatment with the NaCl solution caused significantly higher mortality than the control treatment with the wetting agent alone. Additionally when mortality was expressed as pathogenesis-related mortality, modified blastospores washed with isotonic NaCl solution showed a significantly ($P<0.05$) lower total mortality compared to blastospores washed with water.

In this work modified blastospores with increased levels of the polyols erythritol and mannitol did not show any apparent enhanced mortality compared to unmodified blastospores. This is in contrast to results obtained by Hallsworth & Magan (1994c) who reported that conidia of *B. bassiana*, *P. farinosus* and *M. anisopliae*, primed to contain elevated concentrations of glycerol and erythritol, killed wax moth larvae (*G. mellonella*) faster than conidia cultured on SDA. Pascual *et al.* (1996), also reported that control of peach twig blight (*Monilinia laxa*) in the field was improved by priming conidia of *E. nigrum* by growth on media under water-stress conditions. It is noted here that bioassays were performed under conditions of 100% R.H. Under these conditions no difference in germination capacity between any of the blastospore treatments (unmodified, modified) was observed on water agar media (see Section 3.3.5). On the other hand, there were statistical differences in germination between unmodified and modified blastospores when germination was checked on media with imposed-water stress (Section 3.3.5). A bioassay study under lower R.H. (93-95%) would therefore be very useful to elucidate whether the enhanced germination of primed blastospores with increased levels of polyols *in vitro* leads to enhanced insect mortality. The effect of osmoprotection on modified blastospores pathogenicity should also be examined using an isotonic solution other than NaCl that does not induce high mortality.

Chapter 7 CONCLUSION AND FUTURE WORK

7.1 CONCLUSION

For the successful development of a fungal biocontrol agent as a commercial product several aspects are required to be fulfilled. First of all, a careful screening that will evaluate any potential impact of the candidate biocontrol agent on non-target organisms including vertebrates and humans must be carried out. Once its safety has been well established the following requirements need to be considered.

1. Development of a fermentation system that uses cheap raw materials and produces high numbers of good quality propagules at short fermentation times
2. Enhanced germination efficiency of the desired propagules under conditions of low water availability
3. Enhanced pathogenicity
4. Resistance to drying
5. High stability in storage (more than 18 months and at room temperature)

The present study considered the fulfilment of the above requirements by employing 2 different fermentation systems (solid substrate and liquid) and the entomopathogenic fungus *M. anisopliae* as the model microorganism. The ability of fungi to adapt to different environmental conditions by changing their intracellular composition was exploited by manipulating the cultural environment. Different nutritional factors, stress conditions imposed by water limitations and non-optimum pH conditions were examined for their effect on production and quality of spores. The main findings are summarised below.

Solid substrate fermentation

- The yield and the quality (endogenous polyols, germination efficiency) were greatly influenced by the type of the substrate used and the water availability conditions.
  - ✓ Bulgar wheat supported a higher yield of better quality conidia compared to millet grains.
  - ✓ Under high water-stress conditions the endogenous polyol content of conidia was composed of a higher percentage of low molecular weight polyols (eythritol, arabitol) compared to higher water availability conditions.
  - ✓ Increasing the water-stress conditions resulted in the production of conidia with increased germinability but yield decreased.
  - ✓ Conidial germinability decreased with physiological age.
- Solid substrate fermentation presents a system where manipulation of environmental conditions is very difficult and its heterogeneous nature can often result in inconsistent outcomes. For these reasons it does not provide a system where accurate studies can be carried out. Further work was focused on liquid fermentation as it is a very amenable system to careful environmental manipulation, it requires short fermentation times and it is highly reliable for reproducible outcomes.

Liquid fermentation

- ✓ Increasing water-stress conditions (from 0.998 to 0.96 a_w) increased the incubation time for blastsopore production but increased the highest blastospore production reached compared to unmodified conditions.
- ✓ Blastospore production was dependent on the level of water-stress and modifying-solute and highest production occurred at 0.98 a_w and after 72 h of incubation when PEG 200 was used as the modifying solute.
- ✓ Substantial amounts of endogenous polyols leaked out of modified blastospores (blastospores produced under water-stress conditions) when they were subjected to hypo-osmotic shock post harvest.
- ✓ Osmoprotection of modified blastospores by suspension in isotonic solution after harvest, resulted in retention of higher amounts of polyols and increased

germination for blastospores produced under intermediate waters-stress conditions (0.98 a_w).

- ✓ Under optimised water-stress conditions (0.98 a_w) the combined use of cornsteep solid and yeast extract or cottonseed flour and yeast extract provided the optimum nitrogen status for high blastospore production exceeding $2x10^8$ spores ml^{-1} after 72 h of incubation in the latter case, a yield that it was higher than the spore produced under any condition tested on solid state fermentation in 5 days.
- ✓ Under optimised water-stress (0.98 a_w) and nitrogen source conditions (CS+YE or CF+YE), optimum blastospore production occurred between pH 6.8 and 8.
- ✓ Optimum cultural conditions for production of blastospores with maximum endogenous erythritol, mannitol and total protein occurred at pH 6.8-8 with CF+YE as the nitrogen source and either NaCl or KCl as the a_w-modifying solute.

Enzyme and toxin production

- ✓ Osmoprotection of modified blastospores produced under optimum cultural conditions (0.98 a_w, CS+YE or CF+YE, pH 8) with isotonic PEG 200 solution resulted in increased extracellular protease activity compared to modified blastospores subjected to hypo-osmotic shock and to unmodified blastospores.
- ✓ Optimised modified blastospores produced higher amounts of extracellular protease activity but lower amounts of chitinase activity compared to the best conidial treatment from the solid state fermentation.
- ✓ Osmoprotection of modified blastospores does not inhibit extracellular production of Destruxin A.

Storage stability

- ✓ Osmoprotection of modified blastospores with NaCl isotonic solution confers increased stability in storage as wet pastes and freeze-dried spores.
- ✓ Fluidised bed dried blastospores had either decreased viability (<25%) or good viability (>40%) but high content of moisture (>30%).

Bioassays

- ✓ Modified blastospores with increased levels of polyols did not cause higher mortality in *A. gossypii* compared to unmodified blastospores under 100% R.H.
- ✓ Osmoprotection with NaCl isotonic solution resulted in a significant reduction of total pathogenesis-related mortality compared to washing with water.
- ✓ Modified conidia caused higher mortality in *A. gossypii* compared to both unmodified and modified blastopsores.

Overall finding

An optimised liquid fermentation system using cheap raw materials which supports high yield of *M. anisopliae* blastospores with exceptional germination efficiency under conditions of limited water availability (60-80%) at a very short fermentation time (72 h). Osmoprotection of modified blastospores as a key factor for retention of endogenous reserves and increased efficiency and stability of spores.

7.2 FUTURE WORK

- ✓ More detailed work, including the analysis of a wider range of endogenous compounds like lipids, ions, carbohydrates and biochemical studies about the events that lead to improved germination efficiency of spores produced under different environmental cultural regimes.
- ✓ Elucidation of whether the enhanced germination, protease production and stability of modified blastospores by suspension in isotonic solutions is due to a mere effect of osmoprotection or to an involvement of the additives used for isotonic solution preparation with germination and preservation.
- ✓ Evaluation of potency of modified blastospores and the effect of osmoprotection using an isotonic solution other than NaCl on mortality of insects under R.H. conditions in the range of 93-100%.

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APPENDIX I

Table 1. Analysis of variance of the effect of added glycerol (± gly), water activity (a_w) and time (t), two and three-way interactions on spore production of M. anisopliae grown on bulgar wheat modified to 0.990, 0.980 and 0.960 a_w. The analysis was performed using log_{10} transformed data.

Source	DF	MS	F	P
± gly	1	1.15	121.27	< 0.001 **
a_w	2	0.73	77.01	< 0.001 **
t	2	9.45	994.34	< 0.001 **
± gly *x* a_w	1	0.37	38.54	< 0.001 **
± gly *x* t	2	0.10	10.62	< 0.001 **
a_w *x* t	4	0.03	2.80	0.044 *
± gly *x* a_w *x* t	2	0.0001	0.08	0.925 NS

Note: MS, mean square; ** significant P<0.001; * significant P<0.05; NS, not significant.

Table 2. Analysis of variance of the effect of added glycerol (± gly), water activity (a_w) and time (t), two and three-way interactions on spore production of M. anisopliae grown on millet modified to 0.988, 0.980 and 0.960 a_w. The analysis was performed using log_{10} transformed data.

Source	DF	MS	F	P
± gly	1	3.04	51.36	< 0.001 **
a_w	2	2.38	40.39	< 0.001 **
t	2	8.62	145.83	< 0.001 **
± gly *x* a_w	1	0.84	14.16	< 0.001 **
± gly *x* t	2	0.001	0.00	0.975 NS
a_w *x* t	4	0.25	4.30	0.028 *
± gly *x* a_w *x* t	2	0.10	1.64	0.215 NS

Table 3. Means and least significant difference (LSD) of log_{10} transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to initial inoculum size (0.5ml).

In. size	$5x10^7$	$2x10^7$	$1x10^7$	$5x10^6$	$2x10^6$	$1x10^6$
	6.302	6.523	6.834	6.995	6.940	6.809
LSD						0.078

Table 4. Means and least significant difference (LSD) of log_{10} transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 96h of incubation in liquid culture in relation to initial inoculum size (0.5ml).

In. size	$5x10^7$	$2x10^7$	$1x10^7$	$5x10^6$	$2x10^6$	$1x10^6$
	6.278	6.510	6.826	6.984	6.933	6.837
LSD						0.071

Table 5. Analysis of variance of the effect of a_w, modifying solute, two and three-way interactions on spore production of *M. anisopliae* in liquid culture modified to 0.98, 0.97 and 0.96 a_w with KCl, NaCl or PEG 200. The analysis was performed using log_{10} transformed data.

Source	DF	MS	F	P
Control_v_modified	1	592.86	62.43	< 0.001 **
Control_v_modified *x* a_w	2	2282.40	240.35	< 0.001 **
Control_v_modified *x* solute	2	660.43	69.55	< 0.001 **
Control_v_modified *x* a_w *x* solute	4	553.36	58.27	< 0.001 **

Table 6. Means and least significant difference (LSD) of logit-transformed data of percentage germination of *M. anisopliae* blastospores produced after 72h of incubation in liquid culture modified to 0.98, and 0.97 a_w with KCl or NaCl and washed with water or isotonic solution.

Unmodified /modified	aw	solute	washing	isotonic	water
unmodified	0.988	unmodified		-0.15	-2.40
modified	0.96	KCl		*	*
		NaCl		*	*
	0.97	KCl		-0.88	-2.34
		NaCl		-0.98	-2.05
	0.98	KCl		-0.52	-2.83
		NaCl		-0.69	-2.37
LSD					1.157

Table 7. Means and least significant difference (LSD) of logit-transformed data of percentage germination of *M. anisopliae* blastospores produced after 84h of incubation in liquid culture modified to 0.98, and 0.97 a_w with KCl or NaCl and washed with water or isotonic solution.

Unmodified /modified	aw	solute	washing	isotonic	water
unmodified	0.988	unmodified		-0.22	-0.22
modified	0.96	KCl		*	*
		NaCl		*	*
	0.97	KCl		-0.41	-1.11
		NaCl		-0.63	-0.10
	0.98	KCl		-2.37	-0.53
		NaCl		-2.63	-0.45
LSD					0.629

Table 8. Means and least significant difference (LSD) of logit-transformed data of percentage germination of *M. anisopliae* blastospores produced after 108h of incubation in liquid culture modified to 0.96 a_w with KCl or NaCl and washed with water or isotonic solution.

Unmodified /modified	aw	solute	washing	isotonic	water
unmodified	0.988	unmodified		-1.05	-1.05
modified	0.96	KCl		-1.94	-0.45
		NaCl		-1.36	-0.45
	0.97	KCl		*	*
		NaCl		*	*
	0.98	KCl		*	*
		NaCl		*	*
LSD					0.358

Table 9. Means and least significant difference (LSD) of logit-transformed data of percentage germination of *M. anisopliae* blastospores produced after 120h of incubation in liquid culture modified to 0.96 a_w with KCl or NaCl and washed with water or isotonic solution.

Unmodified /modified	aw	solute	washing	isotonic	water
unmodified	0.988	unmodified		-0.16	-0.16
modified	0.96	KCl		-2.33	0.88
		NaCl		-3.50	-1.15
	0.97	KCl		*	*
		NaCl		*	*
	0.98	KCl		*	*
		NaCl		*	*
LSD					0.529

Table 10. Means and least significant difference (LSD) of $\log_{10}$ transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to cornsteep solid level (%).

CS level	2	4	7
	6.624	7.140	7.273
LSD			0.1678

Table 11. Means and least significant difference (LSD) of $\log_{10}$ transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to cottonseed flour level (%).

CF level	2	4	7
	7.151	7.271	7.619
LSD			0.0748

Table 12. Means and least significant difference (LSD) of log_{10} transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to yeast extract level (%).

YE level	2	4	7
	6.163	6.463	6.514
LSD			0.1645

Table 13. Means and least significant difference (LSD) of log_{10} transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to mycological peptone level (%).

MP level	2	4	7
	6.671	6.689	6.693
LSD			0.2429

Table 14. Means and least significant difference (LSD) of final dry weight of biomass (mg/ml) of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to cornsteep solid level (%).

CS level	2	4	7
	8.39	12.05	15.28
LSD			5.880

Table 15. Means and least significant difference (LSD) of final dry weight of biomass (mg/ml) of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to cottonseed flour level (%).

CF level	2	4	7
	3.81	4.20	14.92
LSD			1.667

Table 16. Means and least significant difference (LSD) of final dry weight of biomass (mg/ml) of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to yeast extract level (%).

YE level	2	4	7
	6.53	6.77	5.49
LSD			0.767

Table 17. Means and least significant difference (LSD) of final dry weight of biomass (mg/ml) of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to mycological peptone level (%).

MP level	2	4	7
	5.8	22.7	41.3
LSD			14.65

Table 18. Means and least significant difference (LSD) of final dry weight of biomass (mg/ml) of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to soy protein level (%).

SP level	2	4	7
	1.66	5.39	0.91
LSD			0.621

Table 19. Means and least significant difference (LSD) of log_{10} transformed data of number of *M. anisopliae* blastospores ml^{-1} produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

nitrogen	pH	solute	KCl	NaCl	PEG
CF	5.00		3.E+07	4.E+07	3.E+07
	6.00		2.E+08	8.E+07	1.E+08
	8.00		2.E+08	1.E+08	1.E+08
	9.00		1.E+08	3.E+07	7.E+07
	10.00		1.E+00	1.E+00	6.E+07
CS	5.00		5.E+06	7.E+05	8.E+06
	6.00		5.E+07	4.E+07	5.E+07
	8.00		5.E+07	5.E+07	8.E+07
	9.00		8.E+07	3.E+07	7.E+07
	10.00		1.E+00	2.E+05	3.E+07
LSD					3.E+07

Table 20. Means and (LSD) of final dry biomass mg ml^{-1} accumulation of *M. anisopliae* produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

nitrogen	pH	solute	KCl	NaCl	PEG
CF	5.00		6.74	8.33	4.13
	6.00		10.76	12.31	10.25
	8.00		10.79	12.18	8.49
	9.00		10.48	6.23	8.09
	10.00		0.00	3.47	3.53
CS	5.00		7.62	10.48	3.12
	6.00		9.64	13.27	8.70
	8.00		7.33	18.27	8.50
	9.00		6.34	13.17	5.07
	10.00		0.00	4.43	5.33
LSD					2.312

Table 21. Means and (LSD) of endogenous total protein mg g^{-1} f.w. in *M. anisopliae* blastospores produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

nitrogen	pH	solute	KCl	NaCl	PEG
CF	5.00		15.29	15.13	13.11
	6.00		19.16	16.62	14.72
	8.00		17.74	14.16	12.50
	9.00		13.70	9.92	9.91
	10.00		13.55	9.79	8.24
CS	5.00		9.89	5.43	7.67
	6.00		13.23	11.01	11.75
	8.00		16.98	11.00	12.40
	9.00		12.82	9.30	11.38
	10.00		14.65	8.57	10.38
LSD					2.753

Table 22. Means and (LSD) of logit-transformed data of percentage germination of *M. anisopliae* blastospores produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

nitrogen	pH	solute	KCl	NaCl	PEG
CF	5.00		-2.373	-2.013	-0.940
	6.00		2.105	0.467	-0.435
	8.00		1.608	0.602	0.297
	9.00		0.513	-0.771	0.728
	10.00		0.583	-0.396	0.327
CS	5.00		-0.820	-6.907	-2.034
	6.00		-0.548	-0.686	-0.394
	8.00		-0.698	-0.215	-0.318
	9.00		0.304	-1.455	0.446
	10.00		0.521	-1.544	0.287
LSD					0.7761

Table 23. Means and (LSD) of germ tube length (□m) data of percentage germination of *M. anisopliae* blastospores produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

nitrogen	pH	solute	KCl	NaCl	PEG
CF	5.00		19.45	15.66	18.38
	6.00		35.64	22.16	20.49
	8.00		33.09	26.81	25.15
	9.00		24.38	20.12	25.11
	10.00		29.41	21.76	25.11
CS	5.00		24.63	0.00	16.04
	6.00		21.09	17.99	22.05
	8.00		19.74	19.16	22.96
	9.00		25.40	18.13	32.18
	10.00		27.53	17.74	27.91
LSD					4.660

Table 24. Means and (LSD) of logit-transformed data of percentage germination under conditions of fully available water after 2 weeks storage of freeze-dried *M. anisopliae* blastospores produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

Unmodified /modified	nitrogen	solute	washing	NaCl	water
unmodified		unmodified			-1.172
modified		conidia			-3.377
	CF	KCl		-0.756	-1.587
		NaCl		-1.640	-1.847
	CS	KCl		-4.595	-4.595
		NaCl		-1.972	-2.448
LSD					0.2912

Table 25. Means and (LSD) of logit-transformed data of percentage germination under water-stress conditions of freeze-dried *M. anisopliae* blastospores produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

Unmodified /modified	nitrogen	solute	washing	NaCl	water
unmodified		unmodified			-0.193
modified		conidia			0.508
	CF	KCl		-0.435	-0.147
		NaCl		0.508	-0.082
	CS	KCl		-1.817	-1.883
		NaCl		0.337	-0.320
LSD					0.6634

Table 26. Means and (LSD) of logit-transformed data of percentage germination under water-stress conditions after 2 weeks of storage of freeze-dried *M. anisopliae* blastospores produced after 72h of incubation in liquid culture in relation to nitrogen source, a_w-modifying solute, and pH.

Unmodified /modified	nitrogen	solute	washing	NaCl	water
unmodified		unmodified			-1.664
modified		conidia			-3.377
	CF	KCl		-2.089	-2.318
		NaCl		-1.121	-3.515
	CS	KCl		-4.595	-4.595
		NaCl		-1.521	-2.642
LSD					0.5161

APPENDIX II

Live *Metarhizium anisopliae* blastospores or conidia were mixed with dead (after autoclaving) ones to make spore suspensions of theoretical percentage values of live spores of 100, 80, 60, 40, 20 and 0 %. The experimental percentage values of live spores were checked using the viability method by Hutcheson *et al.* (1988). The standard curves showing the validity of this method for *M. anisopliae* blastospores and conidia are shown below.

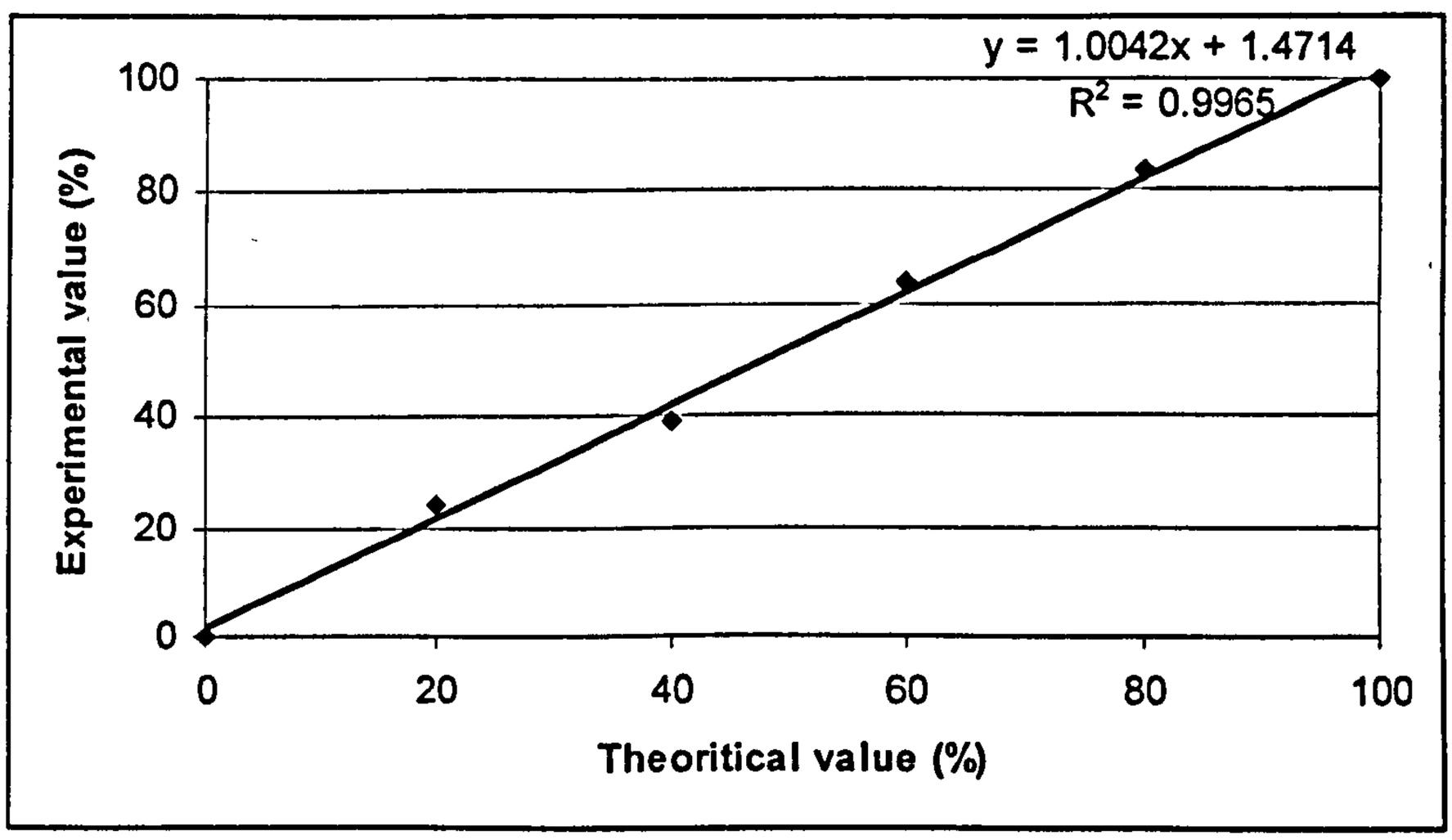

Figure 1. Standard curve of correlation between theoretical and experimental values of percentage viable *M. anisopliae* blastospores.

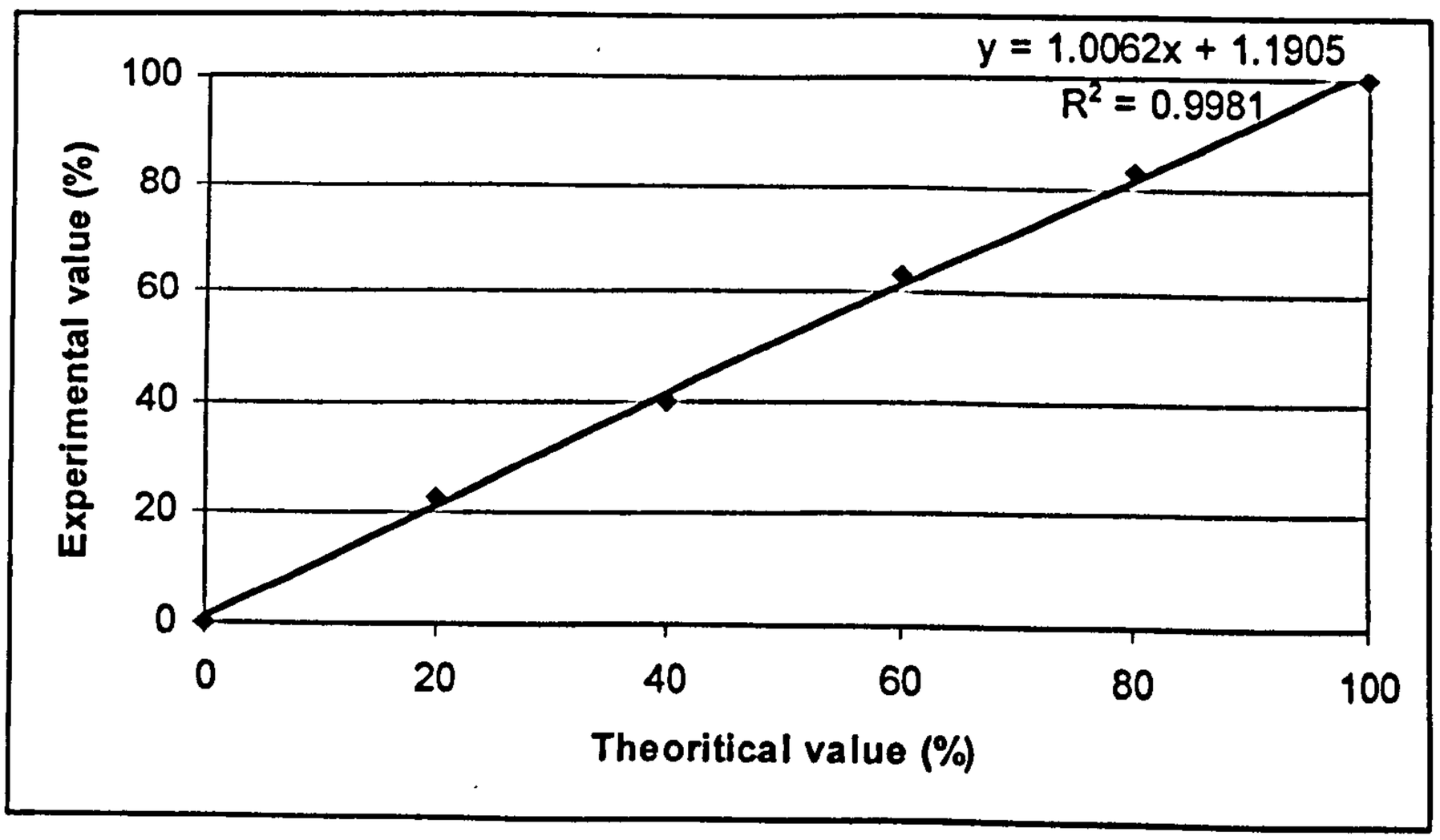

Figure 2. Standard curve of correlation between theoretical and experimental values of percentage viable *M. anisopliae* conidia.

APPENDIX III

II.I PUBLICATIONS

Ypsilos I., Chandler D. & Magan N. 2002. Ecophysiological manipulation of solid and liquid fermentation affects yield and viability of the biocontrol agent *Metarhizium anisopliae*. *Biological Control of Fungal and Bacterial Pathogens, IOBC wrps Bulletin*, Vol. 25 (10) pp.77-80.

Ypsilos I.K. & Magan N. 2004. Impact of water stress and washing treatments on production, synthesis and retention of endogenous sugar alcohols and germinability of *Metarhizium anisopliae* blastospores. *Mycological Research*, in Press

Ypsilos I.K. & Magan N. 2004. Characterisation of optimum cultural environmental conditions for the production of high numbers of *Metarhizium anisopliae* blastospores with enhanced ecological fitness. *Applied and Environmental Microbiology*, Submitted.

II.II ORAL PRESENTATIONS

September 2001, 13th annual meeting of the British Invertebrate Mycopathologists, Warwick, UK
Solid substrate environmental conditions affect production and viability of the entomopathogenic fungus *Metarhizium anisopliae*.

May 2002, Influence of A-Biotic and Biotic Factors on Biocontrol Agents, Kusadasi, TURKEY
Ecophysiological manipulation of solid and liquid fermentation affects yield and viability of the biocontrol agent *Metarhizium anisopliae*.

July 2002, British Mycological Society, Howard Eggins Meeting, Liverpool, UK
Improvement of the ecophysiological competence of the entomopathogenic fungus *Metarhizium anisopliae*.

April 2003, Society for General Microbiology, 152nd meeting, Edinburgh, UK
Production of *Metarhizium anisopliae* blastospores and ecological factors influence quality.

September 2003, 15th annual meeting of the British Invertebrate Mycopathologists, Warwick, UK
Enhancement of *Metarhizium anisopliae* blastospore quality and shelf-life by post-harvest washing treatment.

II.III. POSTER PRESENTATIONS

June 2002, 3rd Postgraduate Research Conference, Cranfiled University, Silsoe, UK
Improvement of the ecophysiological competence of the entomopathogenic fungus *Metarhizium anisopliae*.

October 2002, 11th Panhellenic Symposium of Plant Pathology, Preveza, GREECE
Effect of liquid culture conditions on the production and ecological fitness of the entomopathogenic fungus *Metarhizium anisopliae*.